

PCT/NZ2004/000197

REC'D 14 SEP 2004

WIPO PCT

## CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 22 August 2003 with an application for Letters Patent number 527800 made by ANTIPODEAN BIOTECHNOLOGY LIMITED.

Dated 1 September 2004.

**PRIORITY DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH  
RULE 17.1(a) OR (b)



Neville Harris  
Commissioner of Patents, Trade Marks and Designs



BEST AVAILABLE COPY

BEST AVAILABLE COPY

**NEW ZEALAND PATENTS ACT 1953**

**PROVISIONAL SPECIFICATION**

**MITOCHONDRIALLY TARGETED ANTIOXIDANTS**

We, **ANTIPODEAN BIOTECHNOLOGY LIMITED**, a company duly incorporated under the laws of New Zealand of Level 2, 16 Viaduct Harbour Avenue, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

## MITOCHONDRIALLY TARGETED ANTIOXIDANTS

### TECHNICAL FIELD

The invention relates to antioxidants having a lipophilic cationic group, their synthesis and physicochemical properties that favour their use as, for example, pharmaceuticals.

### BACKGROUND OF THE INVENTION

Oxidative stress contributes to a number of human degenerative diseases associated with ageing, such as Parkinson's disease, and Alzheimer's disease, as well as to Huntington's Chorea and Friedreich's Ataxia, and to non-specific damage that accumulates with aging. It also contributes to inflammation and ischaemic-reperfusion tissue injury in stroke and heart attack, and also during organ transplantation and surgery. To prevent the damage caused by oxidative stress a number of antioxidant therapies have been developed. However, most of these are not targeted within cells and are therefore less than optimally effective. Moreover, many such antioxidants have unfavourable physicochemical properties that limit their bioavailability, and their ability to penetrate to the target organ to exert a therapeutic effect.

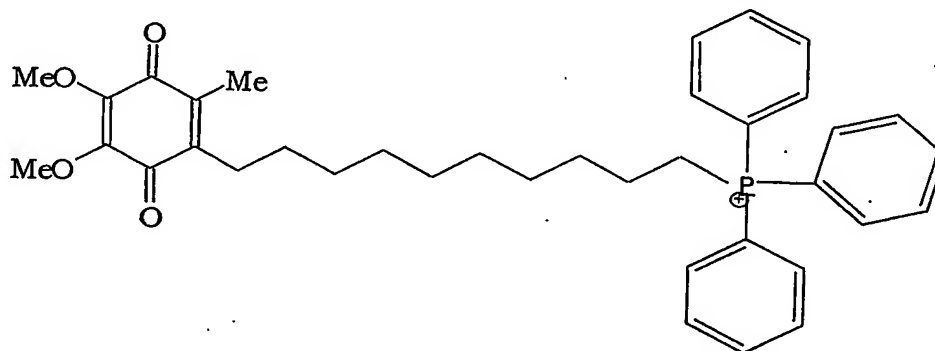
Mitochondria are intracellular organelle responsible for energy metabolism. Consequently, mitochondrial defects are damaging, particularly to neural and muscle tissues which have high energy demands. They are also the major source of the free radicals and reactive oxygen species that cause oxidative stress inside most cells. Therefore, the applicants believe delivering antioxidants selectively to mitochondria will be more effective than using non-targeted antioxidants. Accordingly, it is towards the provision of antioxidants which may be targeted to mitochondria that the present invention is directed.

Lipophilic cations may be accumulated in the mitochondrial matrix because of their positive charge (Rottenberg, 1979 *Methods Enzymol* 55, 547. Chen, 1988 *Ann Rev Cell Biol* 4, 155). Such ions are accumulated provided they are sufficiently lipophilic to screen the positive charge or delocalise it over a large surface area, also provided that there is no active efflux pathway and the cation is not metabolised or immediately toxic to a cell.

The focus of the invention is therefore on an approach by which it is possible to use the ability of mitochondria to concentrate specific lipophilic cations to take up linked antioxidants so as to target the antioxidant to the major source of free radicals and reactive oxygen species causing the oxidative stress.

Examples of antioxidant compounds that show good antioxidant activity yet exhibit poor bioavailability with respect to the target compartment *in vivo* include Coenzyme Q (CoQ) and Idebenone. Both of these compounds must be administered at very high dose rates to be efficacious, and therefore have low therapeutic efficacy when referenced to the dose rate administered.

In US Patent No. 6331532 by reference to exemplifications of compounds mitoquinol and mitoquinone (referred to collectively herein as mitoquinone/mitoquinol) there is disclosed the prospect of mitochondrial targeting of an antioxidant moiety reliant upon a lipophilic cation covalently coupled to the antioxidant moiety. The exemplified compound (1) therein (despite generalisation of the bridge length), is the compound mitoquinone of the formula



(1)

with a carbon bridge length of 10 (i.e., C<sub>10</sub> bridged). Its reduced form, mitoquinol, is also C<sub>10</sub> bridged.

Mitoquinone/mitoquinol, despite excellence in antioxidant activity and targeting and accumulation in mitochondria *in vitro*, we have *in vivo* found to be inappropriate for delivery orally for the treatment of oxidative stress. This is because of the inordinately high dosages that would be required to achieve any appropriate levels of mitochondrial accumulation.

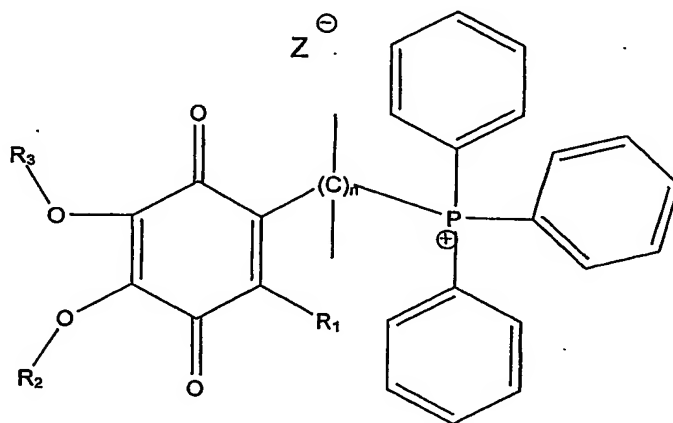
We believe that for an antioxidant compound, activity *in vitro* (whether antioxidant activity, or mitochondrial accumulation) is by no means the sole determinant of efficacy *in vivo* and/or of therapeutic efficacy. Whilst it is true that, to be useful as a mitochondrially-targeted antioxidant compound of the present invention, an antioxidant compound must exhibit a suitably high antioxidant activity *in vitro*, to be efficacious *in vivo* the mitochondrially targeted antioxidant compounds must exhibit other desirable physicochemical properties, for example, high bioavailability.

Paradoxically, we have determined that a mitochondrially targeted compound (such as Mitoquinone-C3 described herein) which *in vitro* exhibits less antioxidant activity than a

compound system such as that of mitoquinone/mitoquinol nonetheless *in vivo* has dosage efficacy greatly in excess of that of mitoquinone/mitoquinol. This will provide the prospect of lower cost dosage units and treatment regimes, better compliance, and likely with fewer dosage related side effects.

- 5 We have determined that mitoquinone (1)/mitoquinol has a high partition coefficient (eg., about 160 when assessed by an octanol:water partition system, see herein), that idebenone has a high partition coefficient of  $3.1 \times 10^3$ , and ubiquinone (CoQ<sub>10</sub>) has a very high partition coefficient of  $1.8 \times 10^{20}$ .

We believe compounds of the general formula I



I

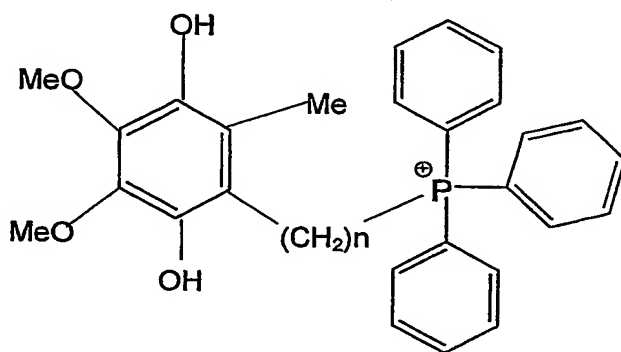
where the bridge length is less than C<sub>10</sub> (and preferably less than C<sub>7</sub>, more preferably less than C<sub>5</sub>) will have low partition coefficients which are in the same order as those of derivatives of CoQ such as CoQ<sub>0</sub>, yet surprisingly provide *in vivo* when orally administered mitochondrial targeting of antioxidant activity many times beyond what would be expected from the *in vitro* studies of mitochondrial targeting of antioxidant activity, and many times beyond that observed with, for example, derivatives of CoQ such as CoQ<sub>0</sub>. By way of example, when the bridge of the mitochondrially targeted antioxidant compound is C<sub>5</sub>, the partition coefficient of the crystalline compound (Mitoquinone-C<sub>5</sub>) is  $2.8 \pm 0.3$ , *i.e.*, much less than that of the C<sub>10</sub> compound, and the level of *in vivo* activity of the C<sub>5</sub> compound (Mitoquinone-C<sub>5</sub>) versus the C<sub>10</sub> compound (mitoquinone/mitoquinol) is several orders of magnitude greater when given

orally and the targeting is to the mitochondria of cells of the internal organs (eg., brain, heart, etc).

We believe without wishing to be bound by any theory that at least in part by virtue of their low partition coefficient the mitochondrially-targeted antioxidant compounds of the present invention exhibit advantageous bioavailability and/or mitochondrial targeting and accumulation *in vivo* and are thereby therapeutically efficacious at low dose rates in comparison to other antioxidant compounds.

A lower partition coefficient is in our view desirable for greater bioavailability particularly where administration is to be oral and/or where there is targeting of the antioxidant compound to mitochondria in the tissues of internal organs (eg., brain, heart or other organs). Conversely, we believe compounds which exhibit high partition coefficients are inappropriate for delivery orally for the treatment of oxidative stress where there is a requirement for oral bioavailability, organ penetration, and for passage through a barrier such as that of the blood brain barrier.

In PCT/NZ02/00154 there is disclosed a process for manufacturing compounds of the general formula II



II

Such a procedure we have found is inappropriate for chain lengths or for bridging groups less than C<sub>6</sub> (ie., where n<6).

The present invention recognises therefore an advantage in being able to prepare compounds of bridge length C<sub>6</sub> or less.

We have found on preparation of compounds of bridge length less than C<sub>6</sub> that such compounds have distinct and desirable physicochemical properties from those taught within US 633152 and PCT/NZ02/00154.

Advantageously, the compounds of the present invention are crystalline and/or solid in form, which amongst other advantages renders them particularly suitable to formulation (eg., by tableting or encapsuling) in orally-administerable dosage forms and pharmaceutical formulations. This, in conjunction with a low partition coefficient (preferably less than about 20, more preferably less than about 10, still more preferably less than about 5), results in novel mitochondrially-targeted antioxidant compounds with desirable physicochemical properties particularly suited to therapeutic pharmaceutical use.

We have also determined a desirability to offer such mitochondrially targeted antioxidant compounds with their positive charge in association with a suitable anion thereby to provide the compound as a general neutralised solid or crystalline product. In such salt forms however certain salt forming anions we have found to be best avoided as they exhibit reactivity against the antioxidant moiety, and/or may lead to cleavage at or of the antioxidant moiety, or are considered pharmaceutically undesirable. For example, nitrate moieties are considered inappropriate generally by pharmaceutical companies as being pharmaceutically or environmentally unacceptable whilst a hydrogen bromide frequently used in salt forming of such compounds we find to have nucleophilic properties that can lead to a reactivity against the antioxidant moiety, for example, a cleavage of a methyl group from the antioxidant moiety of the compound of general formula (2) herein, and/or some overall decrease in stability of the overall compound.

We believe therefore that salt forms as a solid or crystalline form of mitochondrially targeted antioxidants with a bridging (preferably a carbon bridging group) less than six atoms in length (i.e., a C<sub>6</sub> bridging or less) are best associated with an anion or like moiety that is not nucleophilic. It is also preferable that the anion is pharmaceutically acceptable.

## SUMMARY OF THE INVENTION

In its broadest aspect, the invention provides a **mitochondrially-targeted antioxidant compound** which comprises a lipophilic cation covalently coupled to an antioxidant moiety, **wherein**

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

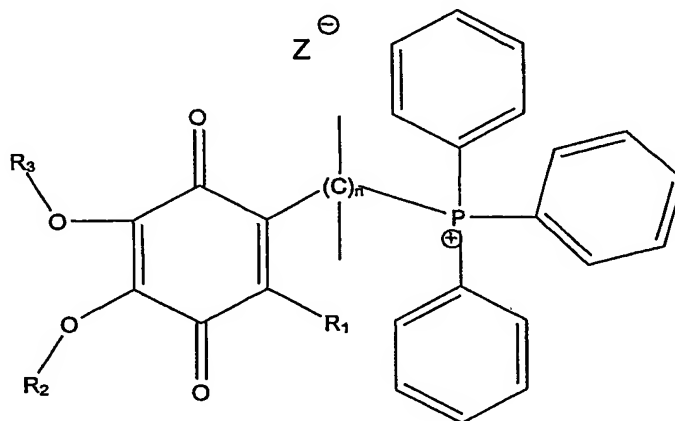
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

- 5 Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

In a second aspect the present invention consists in a **mitochondrially targeted antioxidant compound** of the general formula I



I

wherein R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub>, which can be the same or different, are selected from C<sub>1</sub> to C<sub>5</sub> alkyl (optionally substituted) moieties or H, and wherein n is an integer from 2 to 10, and wherein each C of the (C)<sub>n</sub> bridge is saturated, and wherein Z is an anion, and wherein

- 15 i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical
- 20 preparation, and/or
- v) Z is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.



Conveniently, the antioxidant moiety is a quinone or a quinol.

In a further aspect, the present invention consists in a **dosage unit** suitable for oral administration comprising as an active ingredient a compound in accordance with the present invention, the compound being of a crystalline form and/or non-liquid form.

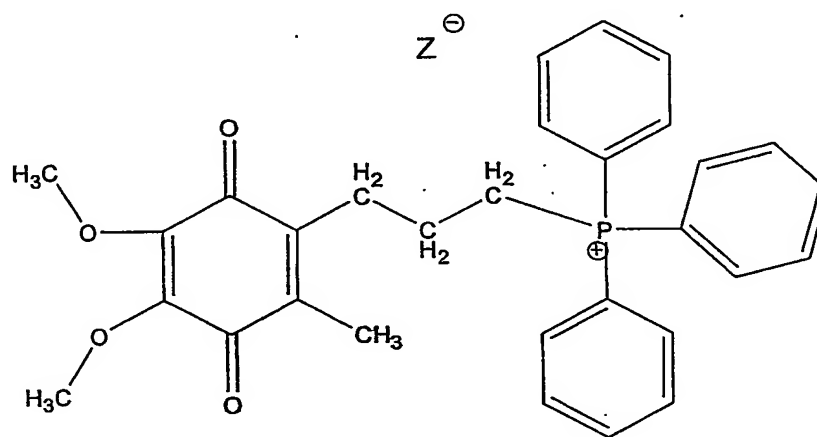
5 Preferably said compound is in the form of a salt.

Preferably said compound has a partition coefficient less than about 10.

Preferably the compound has a bridge of 3 carbon atoms (C<sub>3</sub>).

In a further aspect, the invention provides a mitochondrially-targeted antioxidant compound of the general formula (2)

10



(2)

15 wherein Z is an anion that is not nucleophilic and/or does not exhibit reactivity against the antioxidant moiety, for example but not exclusively alkyl or aryl sulfonates or nitrates.

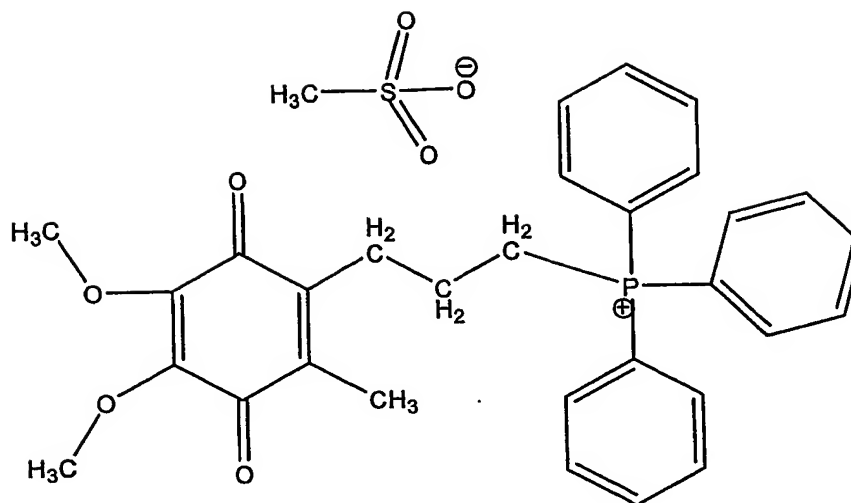
Preferably, Z is a pharmaceutically acceptable anion.

More preferably, Z is an alkyl or aryl sulfonate.

Preferably said compound is in the form of a salt.

20 Preferably said compound has a partition coefficient (octanol:water) less than about 20, more preferably less than about 10.

Most preferably, the mitochondrially-targeted antioxidant has the formula (2)



(2)

In a further aspect, the present invention provides a **pharmaceutical composition** suitable for treatment of a patient who would benefit from reduced oxidative stress which comprises or includes an effective amount of a mitochondrially-targeted antioxidant compound of the present invention in combination with one or more pharmaceutically acceptable carriers or diluents.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

10 More preferably the mitochondrially targeted antioxidant is compound (2).

In a further aspect, the invention provides a **method of reducing oxidative stress in a cell** which comprises the step of administering to said cell a mitochondrially targeted antioxidant compound as defined above.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

15 More preferably the mitochondrially targeted antioxidant is compound (2).

In still a further aspect, the invention provides a **method of therapy or prophylaxis of a patient** who would benefit from reduced oxidative stress which comprises or includes the step of administering to said patient a mitochondrially-targeted antioxidant compound as defined above.

20 Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2).

In a further aspect the invention provides a **pharmaceutical composition** suitable for the treatment of a patient who would benefit from reduced oxidative stress, which comprises an effective amount of a mitochondrially-targeted antioxidant compound comprising a lipophilic

cation covalently coupled to an antioxidant moiety in combination with one or more pharmaceutically acceptable carriers or diluents, and wherein

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2).

In another aspect the invention provides a **pharmaceutical composition** suitable for the treatment of a patient who would benefit from reduced oxidative stress, or reduced symptoms of ageing, which comprises an effective amount of a mitochondrially targeted antioxidant compound as previously described in combination with one or more pharmaceutically acceptable carriers or diluents, and wherein

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2).

In another aspect the invention provides a **pharmaceutical composition** suitable for the treatment of a patient who would benefit from reduced symptoms of aging, which comprises  
 5 an effective amount of a mitochondrially-targeted antioxidant compound comprising a lipophilic cation covalently coupled to an antioxidant moiety in combination with one or more pharmaceutically acceptable carriers or diluents, and wherein

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20,  
 10 and/or
- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- 15 v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

20 Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2) .

In another aspect the invention provides a **method of therapy or prophylaxis of a patient** who would benefit from reduced oxidative stress, or reduced symptoms of ageing which comprises the step of administering to the patient, a mitochondrially-targeted antioxidant  
 25 compound comprising a lipophilic cation covalently coupled to an antioxidant moiety, and **wherein**

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20,  
 and/or
- 30 iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

- v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

- 5 Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2) .

- 10 In another aspect the invention provides a **method of therapy or prophylaxis** of a patient who would benefit from reduced oxidative stress, or reduced symptoms of ageing, which comprises the step of administering to the patient a mitochondrially-targeted antioxidant compound as previously described.

In another aspect the invention provides a **method of reducing oxidative stress in a cell**, which comprises the step of administering to the cell a mitochondrially-targeted antioxidant compound comprising a lipophilic cation covalently coupled to an antioxidant moiety,

- 15 **wherein**

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- 20 iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

- 25 Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2) .

- 30 In another aspect the invention provides a **method of reducing oxidative stress in a cell**, which comprises the step of administering to the cell a mitochondrially-targeted antioxidant as previously described.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2) .

In another aspect the invention provides **the use of a mitochondrially-targeted antioxidant compound** comprising a lipophilic cation covalently coupled to an antioxidant moiety in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use in the reduction of oxidative stress in a patient, **wherein**

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2).

In another aspect the invention provides **the use of a compound as previously described** in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use in for the reduction of oxidative stress in a patient.

In another aspect the invention provides **the use of a mitochondrially-targeted antioxidant compound** comprising a lipophilic cation covalently coupled to an antioxidant in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use in moiety for the reduction of symptoms of aging in a patient, **wherein**

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- 5 v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

- 10 In another aspect the invention provides **the use of a compound as previously described** in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use for the reduction of symptoms of aging in a patient.

- 15 In another aspect the invention provides **the use of a mitochondrially-targeted antioxidant compound** comprising a lipophilic cation covalently coupled to an antioxidant moiety in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use for the reduction of oxidative stress in a cell, **wherein**

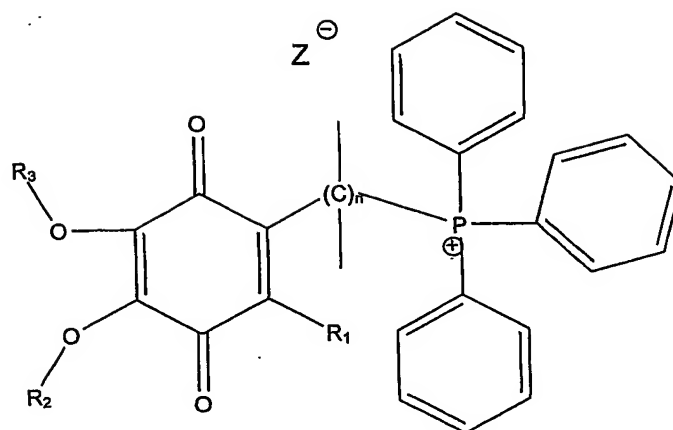
- i) the compound is crystalline or solid, and/or
- 20 ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for
- 25 pharmaceutical preparation, and/or
- v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

- 30 Conveniently, the antioxidant moiety is a quinone or a quinol.

In another aspect the invention provides a **composition** comprising or including a compound of the general formula I



I

herein it is crystalline in form and has a partition coefficient herein defined less than about 20, and/or the compound of the general formula is crystalline in form.

5 In another aspect the present invention provides a **dosage unit** comprising a mitochondrially-targeted antioxidant compound, **wherein**

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- 10 iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- v) the compound is a salt form in which the anion is non nucleophilic, and

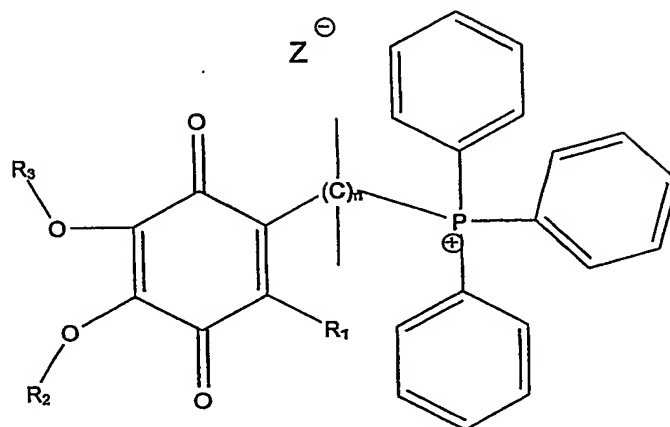
15 **wherein** the unit can be a capsule such as a gelatinized capsule, a tablet, or any other orally-administerable dosage unit.

Preferably an effective amount is present in any such dosage unit such effective amount being related to the dosage regime for the taking of such dosage units for any of the indications herein described.

20 In another aspect the invention provides the **use of a compound as previously described in the preparation or manufacture** with other material or materials of a **medicament, dosage unit, or pharmaceutical composition** effective for use in the reduction of oxidative stress in a cell.



In yet another aspect the present invention consists in a **method of synthesis** of a compound with a moiety or the moiety of the formula I



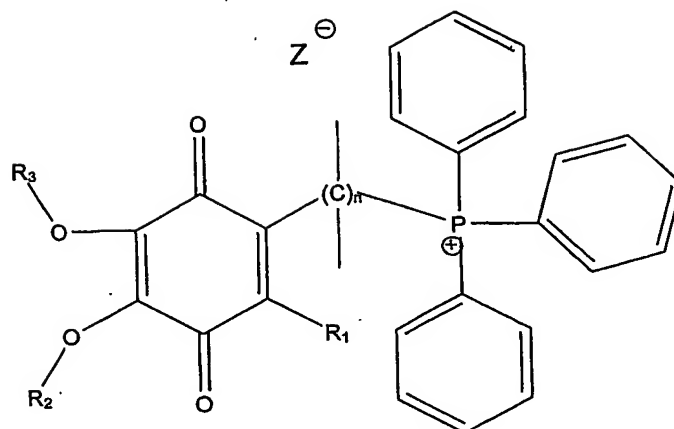
I

(and/or its quinone form) wherein R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub>, which can be the same or different, are selected from C<sub>1</sub> to C<sub>3</sub> alkyl (optionally substituted) moieties, and wherein n is an integer from 2 to 10, and wherein each C of the (C)<sub>n</sub> bridge is saturated, and wherein

- i) the compound is crystalline or solid, and/or
- ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or
- iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or
- iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or
- v) the compound is a salt form in which the anion is non nucleophilic.

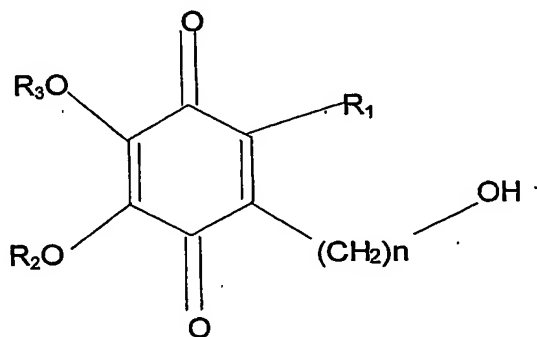
Preferably the formation of the compound from triphenylphosphonium does not involve reaction solvent.

In yet another aspect the present invention consists in a **method of synthesis** of a compound with a moiety or the moiety of the formula I



I

(and/or its quinone form) wherein  $R_1$ ,  $R_2$ , and  $R_3$ , which can be the same or different, are  
 5 selected from  $C_1$  to  $C_3$  alkyl (optionally substituted) moieties, and wherein  $n$  is an integer from 2 to 10, and wherein each C of the  $(C)_n$  bridge is saturated, which comprises or includes the reaction of a compound of the formula IV



IV

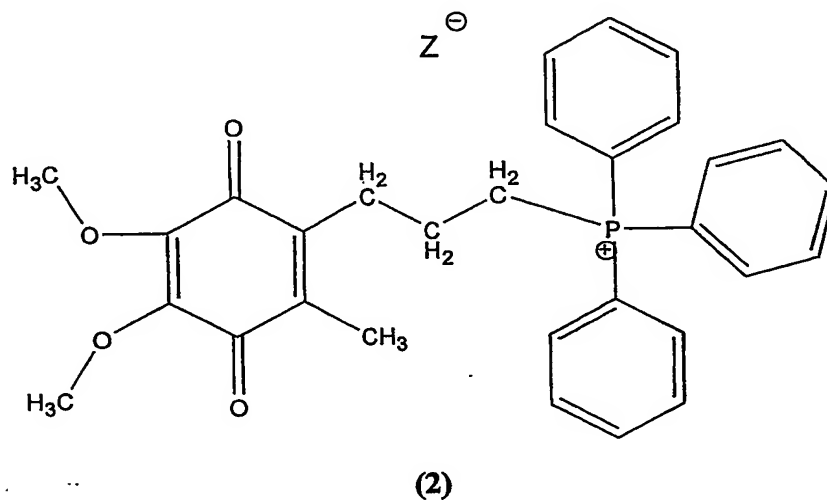
10 (and/or its quinol form) in the presence of  $Ph_3PHX$  and  $Ph_3P$ , where  $X$  is a halogen atom. Preferably  $X$  is preferably bromine, iodine or chlorine (most preferably bromine). Preferably  $n$  is 2 to 5.

More preferably  $n$  is 3.

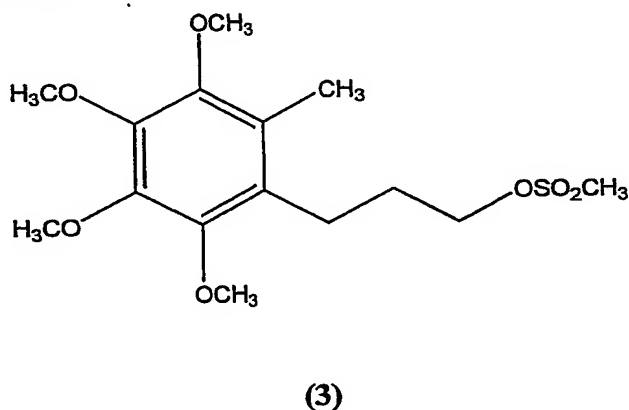
Preferably the reaction is maintained as a temperature below which significant amounts of  
 15  $R_2PPh_3$ , or  $R_3PPh_3$ , are not formed by ether cleavage, eg; the mixture is preferably kept below  $80^\circ C$ .

Preferably the formation of the compound from triphenylphosphonium does not involve reaction solvent.

In yet another aspect the present invention consists in a **method of synthesis** of a compound with a moiety or the moiety of the formula (2)



(and/or its quinol form), wherein Z is an anion, which comprises or includes the reaction of a compound of the formula (3)



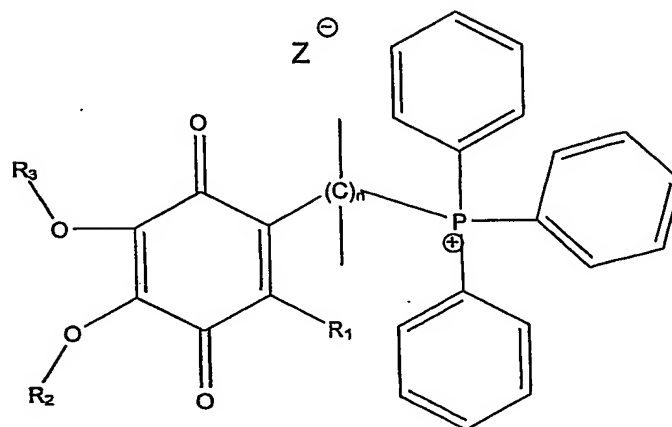
in the presence of  $\text{Ph}_3\text{P}$  and X, where X comprises or includes a halogen atom.

15 Preferably X comprises or includes bromine, iodine or chlorine (most preferably iodine).

Preferably the reaction is maintained as a temperature below which significant amounts of  $\text{MePPh}_3$  are not formed by ether cleavage, eg; the mixture is preferably kept below  $80^\circ\text{C}$ .

Preferably the formation of the compound from triphenylphosphonium does not involve reaction solvent.

In a further aspect the present invention consists in a **method of synthesis** of a compound with a moiety or the moiety of the formula I



I

(and/or its quinone form) wherein  $R_1$ ,  $R_2$ , and  $R_3$ , which can be the same or different, are selected from  $C_1$  to  $C_3$  alkyl (optionally substituted) moieties, and wherein  $n$  is an integer from 2 to 10, and wherein each C of the  $(C)_n$  bridge is saturated, said method substantially as herein described.

- 10 Preferably, said method is reliant upon the method depicted in Scheme 1 herein. More preferably, said method is reliant upon the method depicted in Scheme 1 herein in conjunction with that depicted in Scheme 3 herein.

Preferably the formation of the compound from triphenylphosphonium does not involve reaction solvent.

- 15 Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.
- 20 Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Throughout this specification the term "quinone", whether used alone or prefixed with another term to describe the oxidized form of a compound, will be understood to include within its scope the reduced form of that compound, that is, the quinol form. Similarly, reference to a quinone, by structural depiction for example, also includes within its scope the quinol form.

5 Throughout this specification the term "quinol", whether used alone or prefixed with another term to describe the reduced form of a compound, will be understood to include within its scope the oxidised form of that compound, that is, the quinone form. Similarly, reference to a quinol, by structural depiction for example, also includes within its scope the quinone form. As used herein the term "and/or" includes both "and" and "or" as options.

10 As used herein, the term "partition coefficient" and "partition coefficient (octanol:water)" refer to the octan-1-ol/phosphate buffered saline partition coefficient determined at 25°C or 37°C (see Kelso, G.F., Porteous, C.M., Coulter, C.V., Hughes, G. Porteous, W.K., Ledgerwood, E.C., Smith, R.A.J. and Murphy, M.P. 2001 J Biol Chem 276 4588. Smith, R.A.J., Porteous, C.M., Coulter, C.V. and Murphy, M.P. 1999 Eu. J Biochem 263, 709. 15 Smith, R.A.J., Porteous, C.M., Gane, A.M. and Murphy, M.P. 2003 Proc Nat Acad Sci 100, 5407.), or the octanol/water partition coefficient calculated using Advanced Chemistry Development (ACD) Software Solaris V4.67 as described in Jauslin, M. L., Wirth, T., Meier, T., and Schoumacher, F., 2002, Hum Mol Genet 11, 3055.

As used herein, the phrase "acceptable for pharmaceutical preparation" includes within its 20 meaning not only an acceptability with regard to pharmaceutical administration, but also in respect of formulation for, for example, acceptable stability, shelf life, hygroscopicity, preparation and the like.

Although broadly as defined above, the invention is not limited thereto but also consists of embodiments of which the following description provides examples.

## 25 DESCRIPTION OF DRAWINGS

In particular, a better understanding of the invention will be gained with reference to the accompanying drawings, in which:

Figure 1 is a graph which shows the uptake by isolated mitochondria of compound 4, a mitochondrially-targeted antioxidant according to the present invention;

30 Figure 2 is a graph which shows the accumulation of compound 4 by isolated mitochondria;

Figure 3 is a graph which shows a comparison of a compound 4 uptake with that of the triphenylphosphonium cation (TPMP);

- Figure 4** is a graph which shows that compound 4 protects mitochondria against oxidative damage;
- Figure 5** is a graph which compares compound 4 with vitamin E and the effect of uncoupler and other lipophilic cations;
- 5 **Figure 6** is a graph which shows that compound 4 protects mitochondrial function from oxidative damage;
- Figure 7** is a graph which shows the effect of compound 4 on mitochondrial function;
- Figure 8** is a graph which shows the uptake of compound 4 by cells;
- Figure 9** is a graph which shows the energisation-sensitive uptake of compound 4 by cells;
- 10 **Figure 10** is a graph which shows the effect of compound 4 on cell viability;
- Figure 11** is a graph which shows the UV absorption spectra of mitoquinone (1) and mitoquinol;
- Figure 12** presents graphs showing the reduction of mitoquinone (1) and the oxidation of mitoquinol by complexes of the respiratory chain;
- 15 **Figure 13** presents graphs showing mitoquinone (1) can accept electrons from complex I and complex II of the respiratory chain;
- Figure 14** presents graphs showing mitoquinone (1) can accept electrons within intact mitochondria;
- Figure 15** is a graph which shows the accumulation of mitoquinol within mitochondria;
- 20 **Figure 16** presents graphs showing the effect of mitoquinol on mitochondrial function; and
- Figure 17** is a graph which shows that compounds 1 and 2 protect mitochondria against oxidative damage.

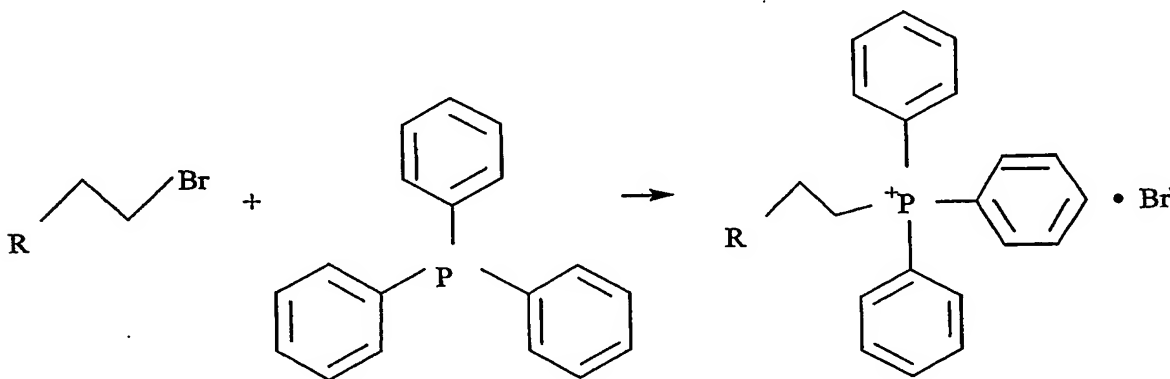
## DESCRIPTION OF THE INVENTION

- 25 As stated above, the focus of this invention is on the mitochondrial targeting of compounds, primarily for the purpose of therapy and/or prophylaxis to reduce oxidative stress.
- Mitochondria have a substantial membrane potential of up to 180 mV across their inner membrane (negative inside). Because of this potential, membrane permeant, lipophilic cations accumulate several-hundred fold within the mitochondrial matrix.
- 30 The applicants have now found that by covalently coupling lipophilic cations (preferably the lipophilic triphenylphosphonium cation) to an antioxidant the compound can be delivered to the mitochondrial matrix within intact cells. The antioxidant is then targeted to a primary

production site of free radicals and reactive oxygen species within the cell, rather than being randomly dispersed.

In principle, any lipophilic cation and any antioxidant can be employed in forming the compounds of the invention. It is however preferred that the lipophilic cation be the triphenylphosphonium cation herein exemplified, and that the lipophilic cation is linked to the antioxidant moiety by a straight chain alkylene group having 1 to 6 carbon atoms; more preferably a propylene group. Other lipophilic cations which may covalently be coupled to antioxidants in accordance with the present invention include the tribenzyl ammonium and phosphonium cations. It will be appreciated by those skilled in the art that moieties other than a straight alkylene may be used to covalently couple the antioxidant moiety to the lipophilic cation, for example, substituted or branched alkyl groups, peptide bonds, and the like.

Such preferred antioxidant compounds can be readily prepared, for example, by the following reaction:



The general synthesis strategy is to heat a precursor containing a suitable leaving group, preferably a alkyl sulfonyl, bromo or iodo precursor with greater than 1 equivalents of triphenylphosphine under argon for several days. The phosphonium compound is then isolated as its salt. To do this the product is triturated repeatedly with diethyl ether until an off-white solid remains. This is then dissolved in chloroform or dichloromethane and precipitated with diethyl ether to remove the excess triphenylphosphine. This is repeated until the solid no longer dissolves in chloroform. At this point the product is recrystallised several times from a suitable solvent such as chloroform, acetone, ethyl acetate or higher alcohols.

It will also be appreciated that the anion of the antioxidant compound thus prepared can readily be exchanged with another pharmaceutically or pharmacologically acceptable

anion, if this is desirable or necessary, using ion exchange or other techniques known in the art.

The applicants have determined that the stability of the salt form of the antioxidant compound is enhanced when the anion is not nucleophilic. It is also desirable that the anion is a pharmaceutically acceptable anion.

Examples of non-nucleophilic anions include hexafluoroantimonate, -arsenate or -phosphate, or tetraphenylborate, tetra(perfluorophenyl)borate or other tetrafluoroborates, trifluoromethane sulfonate, aryl and alkyl sulfonates such as methanesulfonate and p-toluenesulfonate, and phosphates.

Examples of pharmaceutically acceptable anions include halogen ions such as a fluoride ion, chloride ion, bromide ion and iodide ion; anions of inorganic acid salts such as nitrate, perchlorate, sulfate, phosphate, and carbonate; pharmaceutically acceptable anions of lower alkylsulfonic acid salts such as methanesulfonic acid, and ethanesulfonic acid salts; pharmaceutically acceptable anions of arylsulfonic acid salts such as benzenesulfonic acid, 2-naphthalenesulfonic acid and p-toluenesulfonic acid salts; pharmaceutically acceptable anions of organic acid salts such as trichloroacetic acid, trifluoroacetic acid, hydroxyacetic acid, benzoic acid, mandelic acid, butyric acid, propionic acid, formic acid, fumaric acid, succinic acid, citric acid, tartaric acid, oxalic acid, maleic acid, acetic acid, malic acid, lactic acid, and ascorbic acid salts; and pharmaceutically acceptable anions of acidic amino acid salts such as glutamic acid and aspartic acid salts.

In the case of preferred antioxidant compounds of the invention, the halogen anion precursor is exchanged for aryl or alkyl sulphonate anions. Examples include, but are not limited to, benzene sulfonate, p-toluene sulfonate, 2-naphthylene sulphonate, methanesulfonate, ethanesulfonate, propanesulfonate. A particularly preferred anion is the methanesulfonate anion.

The same general procedure can be used to make a wide range of mitochondrially targeted compounds with different antioxidant moieties R attached to the triphenylphosphonium (or other lipophilic cationic) salt. These will include a series of vitamin E derivatives, in which the length of the bridge linking the Vitamin-E function with the triphenylphosphonium salt is varied. Other antioxidants which can be used as R include chain breaking antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, quinols and general radical scavengers such as derivatised fullerenes. In addition, spin traps, which react with free radicals to generate stable free radicals can also be synthesized. These



will include derivatives of 5,5-dimethylpyrroline-*N*-oxide, *tert*-butylnitrosobenzene, *tert*-nitrosobenzene,  $\alpha$ -phenyl-*tert*-butylnitron and related compounds.

Once prepared, the antioxidant compound of the invention, in any pharmaceutically appropriate form and optionally including pharmaceutically-acceptable carriers or additives, will be administered to the patient requiring therapy and/or prophylaxis. Once administered, the compound will target the mitochondria within the cell.

Pharmaceutically acceptable carriers or additives may be chosen so as to enhance the stability of the antioxidant compound, or to enhance its bioavailability.

For example, carrier molecules such as cyclodextrin and derivatives thereof are well known in the art for their potential as complexation agents capable of altering the physicochemical attributes of drug molecules. For example, cyclodextrins may stabilize (both thermally and oxidatively), reduce the volatility of, and alter the solubility of, active agents with which they are complexed. Cyclodextrins are cyclic molecules composed of glucopyranose ring units which form toroidal structures. The interior of the cyclodextrin molecule is hydrophobic and the exterior is hydrophilic, making the cyclodextrin molecule water soluble. The degree of solubility can be altered through substitution of the hydroxyl groups on the exterior of the cyclodextrin. Similarly, the hydrophobicity of the interior can be altered through substitution, though generally the hydrophobic nature of the interior allows accommodation of relatively hydrophobic guests within the cavity. Accommodation of one molecule within another is known as complexation and the resulting product is referred to as an inclusion complex. Examples of cyclodextrin derivatives include sulfobutylcyclodextrin, maltosylcyclodextrin, hydroxypropylcyclodextrin, and salts thereof.

Alternatively, the pharmaceutically appropriate form of antioxidant compound may be formulated so as to enhance the stability and bioavailability of the antioxidant compound. For example, enteric coatings may be applied to tablets to prevent the release of the antioxidant compound in the stomach either to reduce the risk of unpleasant side effects or to maintain the stability of the antioxidant compound which might otherwise be subject to degradation of exposure to the gastric environment. Most polymers that are used for this purpose are polyacids that function by virtue of the fact that their solubility in aqueous medium is pH-dependent, and they require conditions with a pH higher than normally encountered in the stomach.

One preferable type of oral controlled release structure is enteric coating of a solid dosage form. Enteric coatings promote the compounds remaining physically incorporated in

the dosage form for a specified period when exposed to gastric juice. Yet the enteric coatings are designed to disintegrate in intestinal fluid for ready absorption. Delay of absorption is dependent on the rate of transfer through the gastrointestinal tract, and so the rate of gastric emptying is an important factor. For some administrations, a multiple-unit type dosage form, such as granules, may be superior to a single-unit type. Therefore, in one embodiment, antioxidant compounds of the invention may be contained in an enterically coated multiple-unit dosage form. In a more preferable embodiment, the antioxidant compound dosage form is prepared by producing particles having an antioxidant compound -enteric coating agent solid on an inert core material. These granules can result in prolonged absorption of the antioxidant compound with good bioavailability.

Typical enteric coating agents include, but are not limited to, hydroxypropylmethylcellulose phthalate, methacrylic acid-methacrylic acid ester copolymer, polyvinyl acetate-phthalate and cellulose acetate phthalate.

It will be appreciated that for an antioxidant compound of the present invention, as for any drug, activity *in vitro* is by no means the sole determinant of efficacy *in vivo*. The antioxidant activity of the antioxidant compounds of the present invention can be determined by methods such as those described herein using, for example, isolated mitochondria and isolated cells. Whilst it is true that, to be useful as a mitochondrially targeted antioxidant compound of the present invention, an antioxidant compounds must exhibit a suitably high antioxidant activity in such assays, to be efficacious *in vivo* the mitochondrially targeted antioxidant compounds must exhibit other desirable physicochemical properties, for example, high bio availability.

Examples of antioxidant compounds that show good antioxidant activity yet exhibit poor bioavailability with respect to the target compartment *in vivo* include Coenzyme Q (CoQ) and Idebenone. Both of these compounds must be administered at very high dose rates (for example, 0.5 – 1.2g) to obtain minimal clinical effects in human patients.

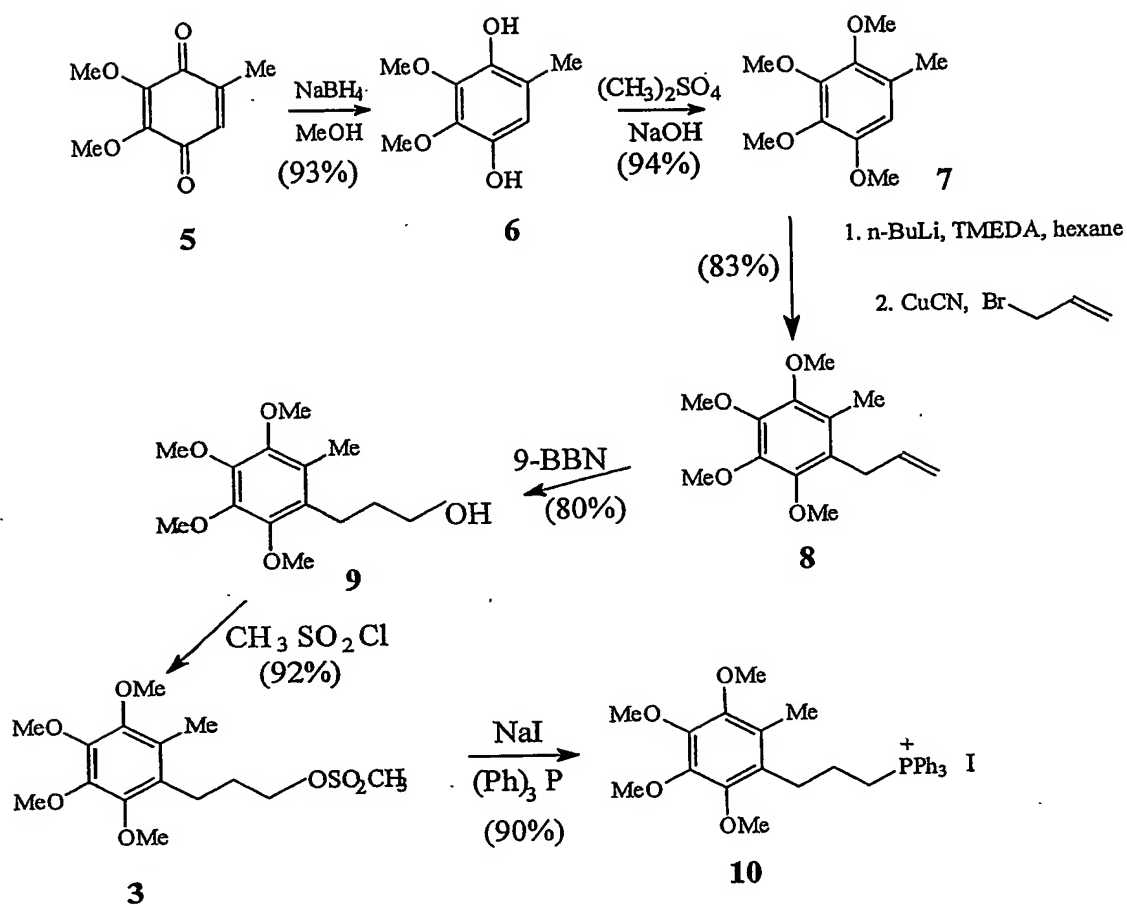
The mitochondrially targeted antioxidant compounds of the present invention exhibit good antioxidant activity and, at least in part by virtue of their low partition co-efficient, excellent bioavailability and thereby are efficacious *in vivo* at low dose rates. We believe the antioxidant compounds of the present invention to be equally effective at mitochondrial targeting, whilst exhibiting the additional benefits of being available as a crystalline or solid form, exhibiting low partition coefficients in turn leading, we believe (without wishing to be bound by any theory), to enhanced bioavailability as compared to the mitochondrially-targeted

antioxidant compounds taught in, for example, US Patent No. 6331532. Such physical and chemical characteristics, we believe, again without wishing to be bound by any theory, confer upon the antioxidant compounds of the present invention preferred characteristics, thereby enabling their use in compositions, formulations and methods amongst other applications to which the antioxidant compounds of the prior art may be less suitable given their chemical and physical properties.

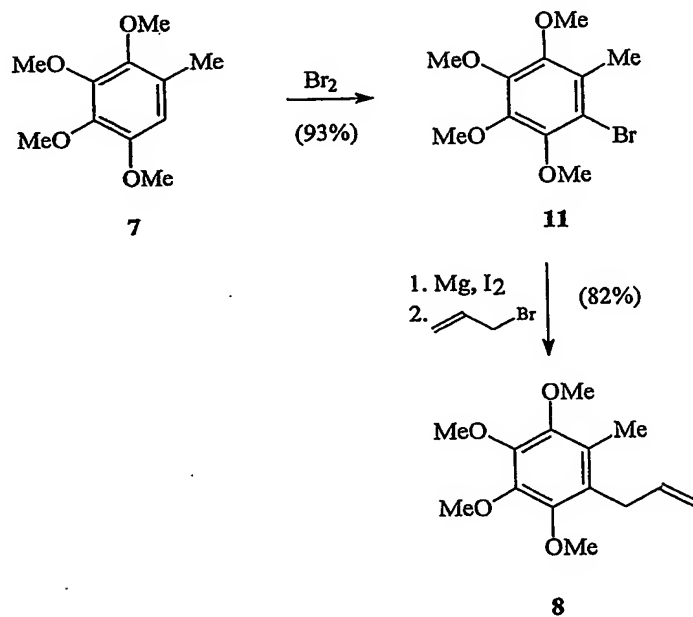
Set out below is the synthetic scheme which may be used to prepare the preferred mitochondrially targeted antioxidant compound of the present invention, namely compound (2).

Preferably the antioxidant compound of the invention will exhibit good bioavailability. Bioavailability is at least in part dependent upon the partition coefficient of the antioxidant compound. Generally, those antioxidant compounds with a low partition coefficient (octanol:water) will be more readily bioavailable than those antioxidant compounds with a high partition coefficient (see table 1). Preferably the antioxidant compounds will have a partition coefficient (octanol:water) that lies in the range of about 0 to about 20, more preferably about 1 to about 10, more preferably a partition coefficient that lies in the range of about 2 to about 5.

An overall synthetic route to compound (2) is shown in Schemes 1 and 3 and is based on converting 4,5-dimethoxy-5-methylbenzoquinone (CoQ<sub>0</sub>) (5) to 2,3,4,5-tetramethoxytoluene (7) then adding a three carbon side chain by metallation and reaction with allyl bromide. Compound (7) has been prepared by alternative routes based on *p*-cresol (Keinan, E.; Eren, D., 1987 *J Org Chem*, **52**, 3872). There are two possible metallation routes: lithiation of 7 followed by copper-catalysed allylation as shown in Scheme 1 or via the bromo derivative (11) followed by Grignard formation and allylation (Yoshioka, T., Nishi, T., Kanai, T., Aizawa, Y., Wada, K., Fujita, T., and Horikoshi, H. 1993 EP 549366. A1 19930630 CAN 119:225944 ) (Scheme 2). Hydroboration of 8 gives the alcohol 9 which is activated as a mesylate (3) and displaced with triphenylphosphine to give the aromatic phosphonium salt 10 iodide. The phosphonium salt formation is best carried out in the absence of solvent using sodium iodide to enhance the reaction.

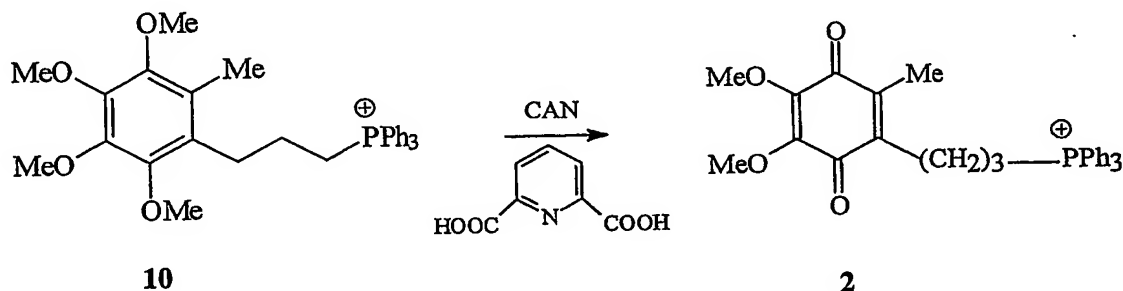


Scheme 1



Scheme 2

Conversion of the tetramethoxyaromatic ring in **10** into the required dimethoxyquinone (**2**) is achieved by using ceric ammonium nitrate (CAN) on the nitrate salt of **10** (Scheme 3). The product is obtained as a nitrate salt and anion exchange gives the target molecule mQ<sub>3</sub> OMs.



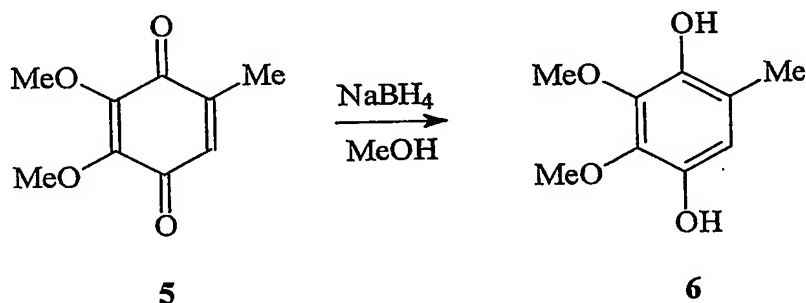
Scheme 3

The invention will now be described in more detail with reference to the following non-limiting experimental section.

#### 10 EXAMPLE 1.

#### Synthesis of a mitochondrially-targeted quinone/ol derivative (Compound 2)

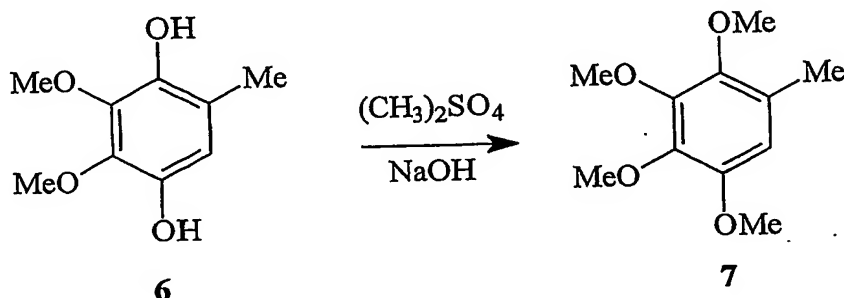
**3,4-Dimethoxy-5-methyl-1,4-benzenediol (6)** (CAS Reg 3066-90-8)



A solution of 2,3-dimethoxy-5-methyl-2,5-cyclohexadiene-1,4-dione (CoQ<sub>0</sub>) (CAS Reg 605-94-7) (**5**) (14.62g, 80.25mmol) in a mixture of ether (220mL) and methanol (109mL) at room temperature was added dropwise to a stirred solution of NaBH<sub>4</sub> (15g, 396 mmol) in H<sub>2</sub>O (440mL). The red color of the oxidize (**5**) was changed to yellow on each addition. After stirring 15 mins at room temperature, the ether phase was separated and the aqueous phase extracted twice with ether (200mL). The combined ethereal extracts were washed with saturated aqueous NaCl (300mL), dried (MgSO<sub>4</sub>), filtered and evaporated *in vacuo* (20mm Hg) to give pure **6** as a yellow liquid (13.65g, 93%).

$^1\text{H}$  NMR (299.9 MHz)  $\delta$  6.48 (1H, s, Ar-H), 3.91, 3.88 (6H, s, Ome), 2.17 (3H, s, Me).

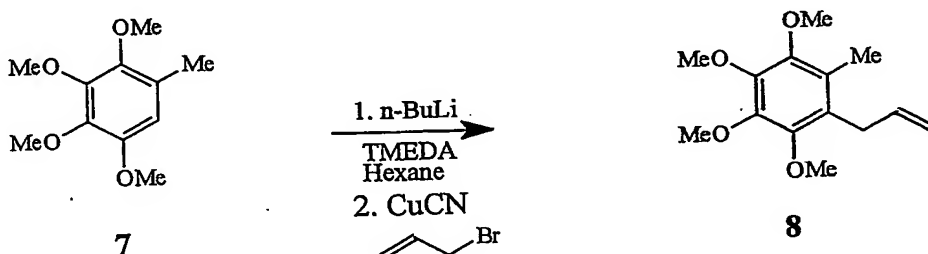
**1,2,3,4-Tetramethoxy-5-methyl-benzene (7)** (CAS Reg 35896-58-3)



- 5 A solution of hydroquinone (**6**) (5.1g, 27.7 mmol) in ethanol (40 mL) was prepared at room temperature and an aqueous solution (40 mL) of NaOH (2.6g) was added in six portions simultaneously with dimethyl sulphate (6mL) with external room temperature water-cooling. After 45 min, 5% HCl (20mL) was added and the mixture was extracted with ethyl acetate (3x200mL). The organic layer was washed successively with water and brine, and dried
- 10 ( $\text{MgSO}_4$ ), filtered and then evaporated *in vacuo* to give a crude product (**7**) as a red oil (5.5g). Column chromatography of the crude product on silica gel (100g, packed in hexane) and elution with 15 % ether in hexane afforded pure **7** as a yellow oil (4.95g, 84 %).
- $^1\text{H}$  NMR (299.9 MHz)  $\delta$  6.44 (1H, s, Ar-H), 3.93, 3.87, 3.82, 3.79 (12H, s, Ome), 2.23 (3H, s, Me).

15

**1,2,3,4-Tetramethoxy-5-methyl-6-(2-propenyl)benzene (8)** (CAS Reg 71573-66-5) *via Lithiation* (Sakamoto, K., Miyoshi, H., Ohshima, M., Kuwabara, K., Kano, K., Akagi, T., Mogi, T., and Iwamura, H. 1998 *Biochemistry* 37 15106).



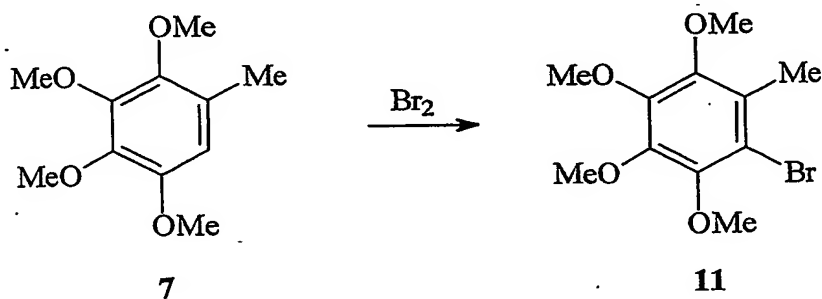
- 20 A solution of **7** (6.35 g, 29.9 mmol) in dry hexane (80 mL) and TMEDA (8.6 mL) was placed with a flame-dried stirrer bar in a flame-dried Schlenk tube under nitrogen. A hexanes solution of n-BuLi (1.6M, 26.2mL) was slowly added at room temperature and the mixture was cooled and stirred at 0° for 1hr. After being cooled to -78 °C, THF (250 mL) was added,

and a small aliquot of the reaction mixture was removed, quenched with D<sub>2</sub>O and examined by <sup>1</sup>H NMR to assure complete metallation. The yellow suspension was then completely transferred to a second flame-dried Schlenk tube containing CuCN (0.54 g) under nitrogen at -78 °C. The mixture was warmed to 0°C for 10 mins, then cooled to -78 °C and allyl bromide (3.62 mL) was added and the reaction was stirred overnight (19hrs) and allowed to warm to room temperature. The reaction was quenched with 10% aqueous NH<sub>4</sub>Cl (75 mL), and extracted with ether (2 x 200 mL). The combined ethereal extracts were washed with H<sub>2</sub>O (2 x 150 mL), 10% aqueous NH<sub>4</sub>OH (200 mL) and saturated aqueous NaCl (200 mL). The organic solvents were dried over MgSO<sub>4</sub>, filtered and the solvent removed by rotary evaporation *in vacuo* to give a crude product (7.25g). The <sup>1</sup>H NMR spectrum of this material indicated >93% product formation.

Column chromatography on silica gel and elution with 10:1 hexane:ethyl acetate gave pure **8** (6.05 g, 83.5 %).

<sup>1</sup>H NMR (299.9 MHz) δ 5.84-5.98 (1H, m, -CH=C), 4.88-5.03 (2H, m, =CH<sub>2</sub>), 3.78, 3.80, 3.90, 3.92 (12H, s, Ome), 3.38 (2H, d, J= Hz, Ar-CH<sub>2</sub>), 2.14 (3H, s, Me).

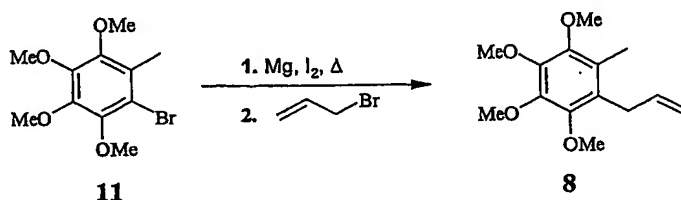
**1-Bromo-2,3,4,5-tetramethoxy-6-methylbenzene (11)** (CAS Reg 73875-27-1)



A solution of Br<sub>2</sub> (7.92 g, 4.9 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was slowly added to a solution of **7** (10g, 4.7 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) at room temperature over 5 mins. The reaction was stirred for a further 5 mins, by which time t.l.c (20% ether:hexane) showed the reaction to be complete. The reaction was quenched with H<sub>2</sub>O (50 mL), then washed with 5% aqueous NaOH (50 mL) and saturated NaCl (50 mL). The organic fraction was dried (MgSO<sub>4</sub>), filtered and the solvent removed *in vacuo* to give the crude product as an orange oil (13.79g). Chromatography on silica gel (180 g) and elution with 10% ether:hexane gave pure **11** (12.72 g, 93%) as a pale yellow oil.

<sup>1</sup>H NMR (299.9 MHz) δ 3.91 (6H, s, Ome), 3.85 (3H, s, Ome), 3.79 (3H, s, Ome), 2.30 (3H, s, Ar-Me).

**1,2,3,4-Tetramethoxy-5-methyl-6-(2-propenyl)benzene (8)** (CAS Reg 71573-66-5) *via Grignard* (Yoshioka, T., Nishi, T., Kanai, T., Aizawa, Y., Wada, K., Fujita, T., and Horikoshi, H. 1993 EP 549366 A1 19930630 CAN 119:225944 )

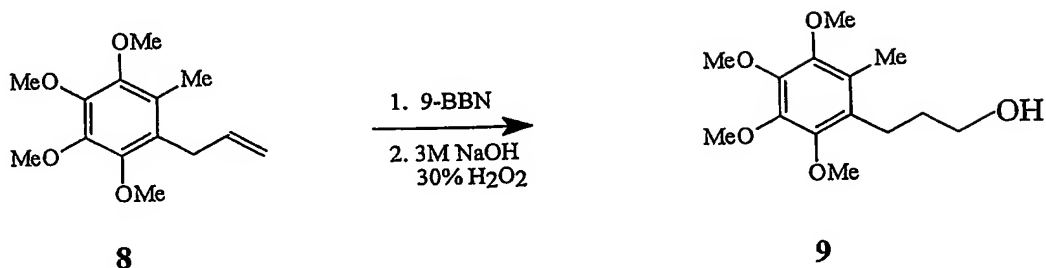


5 Compound **11** (15.26 g, 5.2 mmol) was dissolved in freshly distilled (Na,K) THF (70 mL) in a flame-dried Schlenk tube under argon. Magnesium (1.91 g, 7.8 mmol) was ground in a mortar and pestle and added to another flame-dried Schlenk tube under argon. The Mg was flame-dried under vacuum and, after cooling, a small amount (~20 mg) of Iodine and freshly  
 10 distilled THF (10 mL) was added. The magnesium mixture was stirred for 1 min at 45 °C in which time the solution became turbid and then 10 mL of the THF solution of **11** was added under argon *via* a cannula. The resultant solution quickly lost its turbidity and became pale yellow. The remaining solution of **11** was added in 10 mL portions over 10 mins at 45 °C and then the solution was stirred for a further 20 mins at room temperature. A small aliquot of the  
 15 reaction mixture was quenched at this time with D<sub>2</sub>O and t.l.c (20% ether:hexane) showed all of **11** had been reacted. <sup>1</sup>H NMR indicated 85% deuterium incorporation in the product **7**. Allyl bromide (9 mL, 10.4 mmol) was then added by syringe and the reaction was stirred for 3 hrs at room temperature. The solvent was removed *in vacuo* and saturated aqueous NH<sub>4</sub>Cl (100 mL) was added. The mixture was then extracted with ethyl acetate (4 × 100 mL) and the  
 20 extract dried (MgSO<sub>4</sub>) and filtered. The solvent was removed *in vacuo* to give the crude product as a pale orange oil. Purification by silica gel column chromatography (200g) eluting with 10:1 hexane:ethyl acetate gave **8** (10.30 g, 82 %) as a colourless oil together with a small impure fraction.

<sup>1</sup>H NMR (299.9 MHz) δ 5.84-5.98 (1H, m, CH=C), 4.88-5.03 (2H, m, CH<sub>2</sub>=C), 3.78, 3.80, 3.90, 3.92, (12H, s, Ome), 3.38 (2H, d, Ar-CH<sub>2</sub>-C), 2.14 (3H, s, Ar-Me).  
 25



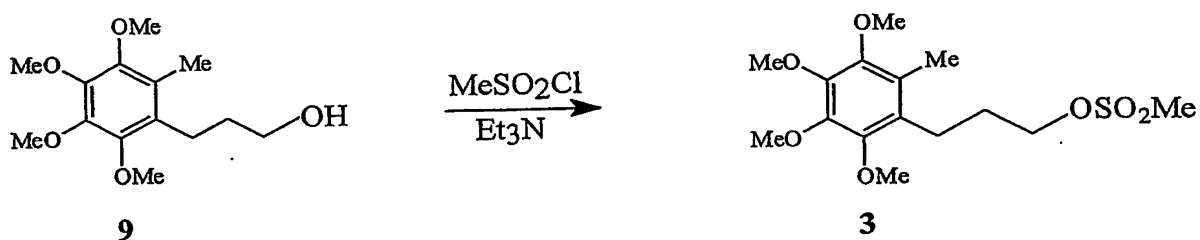
### 3-(2,3,4,5-Tetramethoxy-6-methyl-phenyl)-propan-1-ol (9)



A solution of **8** (8.0 g, 33.05 mmol) in THF (45mL) was added dropwise over 20 mins under argon to a stirred suspension of 9-borabicyclo[3,3,1]nonane (9-BBN) in THF (79mL, 39.67mmol, 0.5M) at 25°. The resulting solution was stirred overnight at room temperature and for further 2hrs at 65° under argon. The mixture was then cooled to 0° and 3M NaOH (53 mL) was then added dropwise followed by 30% aqueous H<sub>2</sub>O<sub>2</sub> (53 mL). After 30 mins stirring at room temperature, the water phase was saturated with NaCl and extracted 3 times with THF. The combined organic fractions were washed with saturated aqueous NaCl, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated to give an oily residue (11.5g) which was purified by column chromatography on silica gel (200 g, packed with ether/hexane 1:9). Elution with ether/hexane 1:4 gave pure **9** as viscous, colourless oil (6.85 g, 80%).

<sup>1</sup>H NMR (299.9 MHz) δ 3.91, 3.90, 3.84, 3.79 (12H, s, OMe), 3.56 (2H, t, J=7.0Hz, -CH<sub>2</sub>-OH), 2.72 (2H, t, J=7.0 Hz, Ar-CH<sub>2</sub>), 2.17 (3H, s, Ar-CH<sub>3</sub>), 1.74 (2H, quintet, J=7.0 Hz, -CH<sub>2</sub>-).

### 1-Methanesulfonyloxy-3-(2,3,4,5-tetramethoxy-6-methyl-phenyl)-propane (3)

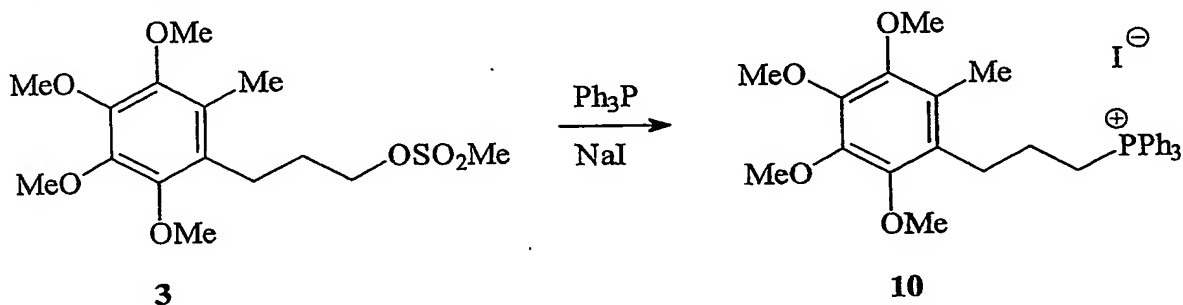


A solution of **9** (3.88 g, 15 mmol) and triethylamine (3.0 g, 30 mmol, 4.2mL) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was stirred at room temperature for 10 mins. Methane sulfonyl chloride (1.8 g, 1.20mL, 15.75 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was added dropwise over 20 mins and the reaction mixture stirred at room temperature for 1 hr. The mixture was then diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and the organic layer was washed with H<sub>2</sub>O (5 x 100 mL), 10% aqueous NaHCO<sub>3</sub> (100 mL), dried

(MgSO<sub>4</sub>), filtered and the solvent removed *in vacuo* by rotary evaporation to afford pure **3** as a liquid (4.8 g, 95 %).

<sup>1</sup>H NMR (299.9 MHz) δ 4.27 (2H, t, *J* = 7.0 Hz, -CH<sub>2</sub>-O-SO<sub>2</sub>-Me), 3.91, 3.89, 3.82, 3.78 (12H, s, Ome), 3.03 (3H, s, -O-SO<sub>2</sub>-Me), 2.70 (2H, t, *J* = 7.0 Hz, Ar-CH<sub>2</sub>-), 2.17 (3H, s, Me),  
5 1.9 (2H, m, -CH<sub>2</sub>-).

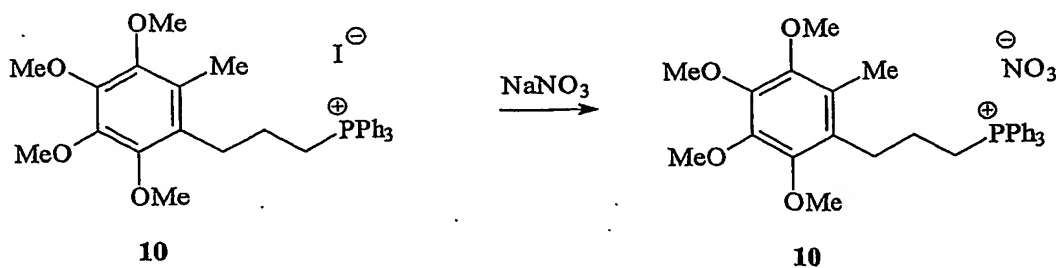
**[3-(2,3,4,5-Tetramethoxy-6-methyl-phenyl)-propyl]triphenylphosphonium iodide (10)**



The methanesulfonate **3** (3.30 g, 9.8 mmol) was mixed with a freshly ground mixture of triphenylphosphine (4.08 g, 15.6 mmol) and NaI (7.78 g, 51.9 mmol) in a KIMAX tube and sealed under argon. The mixture was then held at 70-74 °C with magnetic stirring for 3 hrs during which time the mixture changed from a molten thick liquid into a glassy solid. The tube was cooled to room temperature and the residue stirred with CH<sub>2</sub>Cl<sub>2</sub> (30 mL). The suspension was then filtered and the filtrate evaporated *in vacuo*. The residue was dissolved in a minimum amount of CH<sub>2</sub>Cl<sub>2</sub> and triturated with excess ether (250 mL) to precipitate the white solid. The solid was filtered and washed with ether, dried *in vacuo* to give pure **10** (5.69 g, 90%).

<sup>1</sup>H NMR (299.9 MHz) δ 7.82-7.65 (15H, m, Ar-H), 3.88, 3.86, 3.74, 3.73 (12H, s, Ome), 3.76-3.88 (2H, m, CH<sub>2</sub>-P<sup>+</sup>), 2.98 (2H, t, *J*=7.0 Hz, CH<sub>2</sub>-Ar), 2.13 (3H, s, Ar-CH<sub>3</sub>), 1.92-1.78 (2H, m, -CH<sub>2</sub>-). <sup>31</sup>P NMR (121.4 MHz) δ 25.32 (-CH<sub>2</sub>-P<sup>+</sup>Ph<sub>3</sub>).

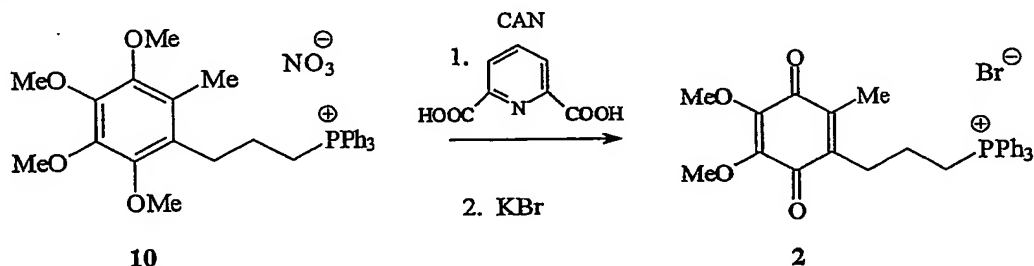
**[3-(2,3,4,5-Tetramethoxy-6-methyl-phenyl)-propyl]triphenylphosphonium nitrate (10)**



A solution of the iodide form of **10** (4.963 g, 7.8 mmol) in  $\text{CH}_2\text{Cl}_2$  (80 mL) was shaken with 10% aqueous  $\text{NaNO}_3$  (50 mL) in a separatory funnel for 5 mins. The organic layer was separated, dried ( $\text{Na}_2\text{SO}_4$ ), filtered and evaporated *in vacuo* to give the crude nitrate form of **10** (4.5 g, 100%).

5

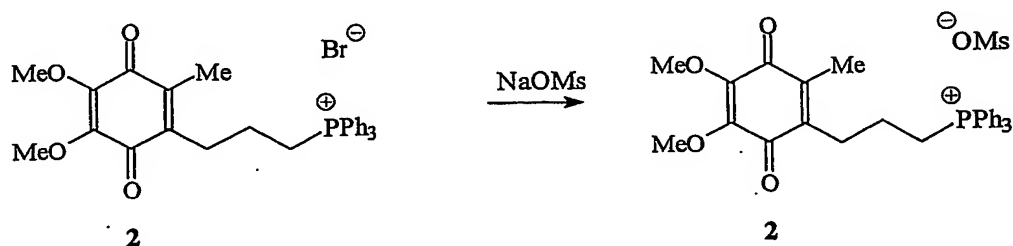
**[3-(4,5-Dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)propyl]triphenylphosphonium bromide (2)**



The nitrate form of **10** (4.5 g, 7.8 mmol) was dissolved in a mixture of  $\text{CH}_3\text{CN}$  and  $\text{H}_2\text{O}$  (7:3, 38 mL) and stirred at  $0^\circ\text{C}$  in an ice bath. Pyridine-2,6-dicarboxylic acid (6.4 g, 39 mmol) was then added followed by drop wise addition of a solution of ceric ammonium nitrate (21.0 g, 39 mmol) in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1, 77 mL) over 5 mins. The reaction mixture was stirred at  $0^\circ\text{C}$  for 20 mins and then at room temperature for a further 10 mins. The reaction mixture was then poured into  $\text{H}_2\text{O}$  (200 mL) and extracted with  $\text{CH}_2\text{Cl}_2$  (200 mL), dried ( $\text{Na}_2\text{SO}_4$ ), filtered and evaporated *in vacuo* to give a crude **2** nitrate. The total product was dissolved in  $\text{CH}_2\text{Cl}_2$  (100 mL) and shaken for 10 mins with 20% aqueous  $\text{KBr}$  (50 mL). The organic layer was separated, dried and evaporated *in vacuo* to give NMR pure **2** bromide (4.1 g, 93.6%).

$^1\text{H}$  NMR (299.9 MHz)  $\delta$  7.90-7.65 (15H, m, Ar-H), 4.15-4.05 (2H, m,  $\text{CH}_2\text{-P}^+$ ), 3.96, 3.95, (6H, s, Ome), 2.93 (2H, t,  $J=7.0$  Hz,  $\text{CH}_2\text{-Ar}$ ), 2.15 (3H, s, Ar- $\text{CH}_3$ ), 1.85-1.70 (2H, m,  $-\text{CH}_2-$ ).  $^{31}\text{P}$  NMR (121.4 MHz)  $\delta$  25.29 ( $-\text{CH}_2\text{-P}^+\text{Ph}_3$ ).

**[3-(4,5-Dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)propyl]triphenylphosphonium methane sulfonate (2)**



A solution of **2** bromide (3.65g, 6.5 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (75 mL) was shaken with an aqueous solution of sodium methane sulphonate (100 mL) in a separatory funnel for 5 mins. The CH<sub>2</sub>Cl<sub>2</sub> layer was separated, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated *in vacuo* to give the methane sulfonate salt of **2** (3.7 g, 98%).

<sup>1</sup>H NMR (299.9 MHz) δ 7.88-7.60 (15H, m, Ar-**H**), 3.93, 3.92, (6H, s, Ome), 3.90-3.78 (2H, m, CH<sub>2</sub>-P<sup>+</sup>), 2.85 (2H, t, J=7.0 Hz, CH<sub>2</sub>-Ar), 2.70 (3H, s, OSO<sub>2</sub>CH<sub>3</sub>), 2.09 (3H, s, Ar-CH<sub>3</sub>), 1.82-1.68 (2H, m, -CH<sub>2</sub>-). <sup>31</sup>P NMR (121.4 MHz) δ 25.26 (-CH<sub>2</sub>-P<sup>+</sup>Ph<sub>3</sub>).

## EXAMPLE 2. Properties of mitochondrially-targeted antioxidant compounds of the present invention

The present invention recognises that, in order to be suitable in a wide variety of applications, for example the formulation of dosage forms such as tablets, there is advantage in being able to form a crystalline or solid form of the mitochondrially-targeted antioxidant compound. Similarly, it is believed, without wishing to be bound by any theory, that mitochondrially-targeted antioxidant compounds with low partition coefficients (octanol:water) may exhibit enhanced bioavailability with respect to those compounds having higher partition coefficients, such that a low partition coefficient is desirable for certain applications.

The partition coefficients for a variety of antioxidant compounds are shown in Table 1. Of particular note is the low partition coefficient of compounds with small numbers of carbon atoms bridging the antioxidant component and the phosphonium. For example, a compound within the present invention, herein referred to as Mitoquinone-C5, which has a 5 carbon bridge has a partition coefficient approximately 50-fold lower than that observed for the related compound, Mitoquinone-C10 (Table 1).

**TABLE I. Partition Coefficients of Coenzyme Q, Targeted Derivatives and Related Compounds**

Compound	Partition coefficient
Methyltriphenylphosphonium (TPMP)	<sup>a</sup> 0.35 ± 0.02
MitoVit E	<sup>b</sup> 7.4 ± 1.6
4-Bromobutyltriphenylphosphonium	<sup>b</sup> 3.83 ± 0.22
4-Iodobutyltriphenylphosphonium	<sup>c</sup> 4.0 ± 0.4
Mitoquinone-C10	<sup>a</sup> 160 ± 9
Mitoquinone-C5	<sup>c</sup> 2.8 ± 0.3

$\alpha$ -Tocopherol	<sup>b</sup> 27.4 $\pm$ 1.9
Bromodecylubiquinone	<sup>d</sup> 310 $\pm$ 60
Idebenone	<sup>d</sup> 3.1 $\times 10^3$
Decylubiquinone	<sup>d</sup> 3.1 $\times 10^5$
Coenzyme Q <sub>0</sub>	<sup>d</sup> 1.33
Coenzyme Q <sub>1</sub>	<sup>d</sup> 409
Coenzyme Q <sub>2</sub>	<sup>d</sup> 4.44 $\times 10^4$
Ubiquinone (Coenzyme Q <sub>10</sub> )	<sup>d</sup> 1.82 $\times 10^{20}$
Ubiquinol	<sup>d</sup> 4.53 $\times 10^{20}$
Decylubiquinol	<sup>d</sup> 7.91 $\times 10^5$
Idebenol	<sup>d</sup> 7.82 $\times 10^3$

Data<sup>a-c</sup> are octan-1-ol/phosphate buffered saline partition coefficients determined at 25°C or 37°C, or octanol/water partition coefficients<sup>d</sup> calculated using Advanced Chemistry Development (ACD) Software Solaris V4.67 as described in Jauslin, M. L., Wirth, T., Meier,

5 T., and Schoumacher, F., 2002, *Hum Mol Genet* **11**, 3055.

<sup>a</sup> Kelso, G.F., Porteous, C.M., Coulter, C. V., Hughes, G., Porteus, W. K., Ledgerwood, E. C., Smith, R. A. J., and Murphy, M. P., 2001, *J Biol Chem* **276**, 4588.

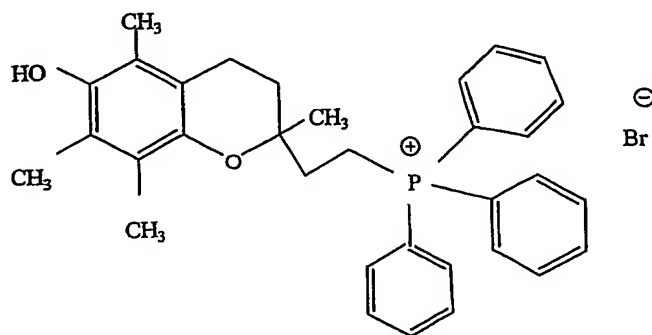
<sup>b</sup> Smith, R. A. J., Porteous, C. M., Coulter, C. V., and Murphy, M. P. 1999 *Eur J Biochem* **263**, 709.

10 <sup>c</sup> Smith, R.A.J., Porteous, C.M., Gane, A.M., and Murphy, M.P. 2003 *Proc Nat Acad Sci* **100**, 9, 5407.

### EXAMPLE 3.

#### Mitochondrial uptake of mitochondrially-targeted compounds

15 To demonstrate that mitochondrial targeting is effective, the exemplary vitamin E compound, compound (4), was tested in relation to both isolated mitochondria and isolated cells.



(4)

To do this a [ $^3\text{H}$ ]-version of compound (4) was synthesized using [ $^3\text{H}$ ]-triphenylphosphine and the mitochondrial accumulation of compound (4) quantitated by scintillation counting (Figure 1) (Burns *et al.*, 1995, *Arch Biochem Biophys* 332, 60. Burns and Murphy, 1997, *Arch Biochem Biophys* 339, 33). To do this rat liver mitochondria were incubated under conditions known to generate a mitochondrial membrane potential of about 180 mV (Burns *et al.*, 1995, *Arch Biochem Biophys* 332, 60. Burns and Murphy, 1997, *Arch Biochem Biophys* 339, 33). Under these conditions compound 4 was rapidly ( $< 10$  s) taken up into mitochondria with an accumulation ratio of about 6,000. This accumulation of compound 4 into mitochondria was blocked by addition of the uncoupler FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) which prevents mitochondria establishing a membrane potential (Figures 1 and 2) (Burns *et al.*, 1995, *Arch Biochem Biophys* 332, 60.). Therefore compound 4 is rapidly and selectively accumulated into mitochondria driven by the mitochondrial membrane potential and this accumulation results in a concentration of the compound within mitochondria several thousand fold higher than in the external medium. This accumulation is rapidly ( $< 10$  s) reversed by addition of the uncoupler FCCP to dissipate the mitochondrial membrane potential after accumulation of compound 4 within the mitochondria. Therefore the mitochondrial specific accumulation is solely due to the mitochondrial membrane potential and is not due to specific binding or covalent interaction.

The mitochondrial specific accumulation of compound 4 also occurs in intact cells. This was measured as described by Burns and Murphy, 1997, *Arch Biochem Biophys* 339, 33 and the accumulation was prevented by dissipating both the mitochondrial and plasma membrane potentials. In addition, compound 4 was not accumulated by cells containing defective mitochondria, which consequently do not have a mitochondrial membrane potential. Therefore the accumulation of compound 4 into cells is driven by the mitochondrial membrane potential.

The accumulation ratio was similar across a range of concentrations of compound 4 and the amount of compound 4 taken inside the mitochondria corresponds to an intramitochondrial concentration of 4-8 mM (Fig 2). This uptake was entirely due to the membrane potential and paralleled that of the simple triphenylphosphonium cation TPMP over a range of membrane potentials (Fig 3). From comparison of the uptake of TPMP and compound 4 at the same membrane potential we infer that within mitochondria about 84% of compound 4 is membrane-bound. Further details of the experimental procedures and results are given below.

Figure 1 shows the uptake of 10  $\mu\text{M}$  [ $^3\text{H}$ ] compound 4 by energised rat liver mitochondria (continuous line and filled symbols). The dotted line and open symbols show the effect of addition of 333 nM FCCP at 3 min. Incubation with FCCP from the start of the incubation led to the same uptake as for adding FCCP at 3 min (data not shown). Liver mitochondria were prepared from female Wistar rats by homogenisation followed by differential centrifugation in medium containing 250 mM sucrose, 10 mM Tris-HCL (pH 7.4) and 1mM EGTA and the protein concentration determined by the biuret assay using BSA as a standard. To measure [ $^3\text{H}$ ] compound 4 uptake mitochondria (2 mg protein/ml) were suspended at 25°C in 0.5 – 1 ml 110 mM KCl, 40 mM Hepes-KOH, pH 7.2, 0.1 mM EDTA supplemented with nigericin (1  $\mu\text{g}/\text{ml}$ ), 10 mM succinate, rotenone 1.33  $\mu\text{g}/\text{ml}$  and 60 nCi/ml [ $^3\text{H}$ ] compound V and 10  $\mu\text{M}$  compound 4. After the incubation mitochondria were pelleted by centrifugation and the [ $^3\text{H}$ ] compound 4 in the supernatant and pellet quantitated by  $\square\text{xidize}\square\text{tion}$  counting.

Figure 2 shows the mitochondrial accumulation ratios [(compound 4/mg protein)/(compound 4/ $\mu\text{l}$ )] obtained following 3 min incubation of energised rat liver mitochondria with different concentrations of compound 4 (filed bars) and the effect of 333 nM FCCP on these (open bars). The dotted line and open circles show compound 4 uptake by mitochondria, corrected for FCCP-insensitive binding. To measure [ $^3\text{H}$ ] compound 4 accumulation ratio mitochondria (2 mg protein/ml) were suspended at 25°C in 0.5 – 1 ml 110 mM KCl, 40 mM Hepes-KOH, pH 7.2, 0.1 mM EDTA supplemented with nigericin (1  $\mu\text{g}/\text{ml}$ ), 10mM succinate, rotenone 1.33  $\mu\text{g}/\text{ml}$  and 6 – 60 nCi/ml [ $^3\text{H}$ ] compound 4 and 1-50  $\mu\text{M}$  compound 4. After the incubation mitochondria were pelleted by centrifugation and the [ $^3\text{H}$ ] compound 4 in the supernatant and pellet quantitated by scintillation counting.

Figure 3 shows a comparison of compound 4 uptake with that of TPMP at a range of mitochondrial membrane potentials. Energised rat liver mitochondria were incubated for 3 min with 10  $\mu\text{M}$  compound 4 and 1  $\mu\text{M}$  TPMP and different membrane potentials established with 0-8 mM malonate or 333 nM FCCP. The accumulation ratios of parallel incubations with either 60 nCi/ml [ $^3\text{H}$ ] compound 4 or 50 nCi/ml [ $^3\text{H}$ ] TPMP were determined, and the accumulation ratio for compound 4 is plotted relative to that of TPMP at the same membrane potential (slope = 2.472, y intercept = 319,  $r = 0.97$ ). Mitochondria (2 mg protein/ml) were suspended at 25°C in 0.5-1 ml 110 mM KCl, 40 mM Hepes-KOH, pH 7.2, 0.1 mM EDTA supplemented with nigericin (1 $\mu\text{g}/\text{ml}$ ), 10 mM succinate, rotenone 1.33  $\mu\text{g}/\text{ml}$ .

**EXAMPLE 4.****Anti-oxidant efficacy of compound 4**

The compounds of the invention are also highly effective against oxidative stress. To demonstrate this, exemplary compound 4 was further tested using rat brain homogenates. The rat brain homogenates were incubated with or without various concentrations of the test compounds (compound 4; native Vitamin E ( $\alpha$ -tocopherol), bromobutyl triphenylphosphonium bromide, Trolox (a water soluble form of Vitamin E) and 2-(2-bromoethyl)-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol, the precursor of compound 4 ("Brom Vit E")) and the oxidative damage occurring over the incubation was quantitated using the TBARS assay (Stocks *et al.*, 1974, *Clin Sci Mol Med* 47,215). From this the concentration of compound required to inhibit oxidative damage by 50% was determined. In this system 210 nM compound 4 inhibited oxidative stress by 50% while the corresponding value for native Vitamin E was 36 nM. The value for bromobutyltriphenylphosphonium bromide, which contains the triphenylphosphonium moiety but not the antioxidant Vitamin E moiety was 47  $\mu$ M. These data show that compound 4 is an extremely effective antioxidant, within an order of magnitude as effective as Vitamin E. Comparison with bromobutyltriphenylphosphonium bromide shows that the antioxidant capability is due to the Vitamin E function and not to the phosphonium salt. Further details of the experimental procedures and results are set out below.

The  $IC_{50}$  values for inhibition of lipid peroxidation were determined in rat brain homogenates, and are means  $\pm$  SEM or range of determinations on 2-3 brain preparations. Octan-1-ol/PBS partition coefficients are means  $\pm$  SEM for three independent determinations. N.D. not determined. Partition coefficients were determined by mixing 200  $\mu$ M of the compound in 2 ml water-saturated octanol-1-ol with 2 ml octanol-saturated-PBS at room temperature for 1 h, then the two layers were separated by brief centrifugation and their concentrations determined spectrophotometrically from standard curves prepared in PBS or octanol. To measure antioxidant efficacy four rat brains were homogenised in 15 ml 40 mM potassium phosphate (pH 7.4), 140 mM NaCl at 4°C, particulate matter was pelleted (1,000 x g at 4°C for 15 min) and washed once and the combined supernatants stored frozen. Aliquots were rapidly thawed and 5 mg protein suspended in 800  $\mu$ l PBS containing antioxidant or ethanol carrier and incubated at 37°C for 30 min. Thiobarbituric acid reactive species (TBARS) were quantitated at 532 nm by adding 200  $\mu$ l conc.  $HClO_4$  and 200  $\mu$ l 1%



thiobarbituric acid to the incubation, heating at 100°C for 15 min and then cooling and clarification by centrifugation (10,000 x g for 2 min). The results are shown in Table 2 below.

**Table 2. Partition coefficients and antioxidant efficacy of compound 4 and related compounds**

Compound	IC <sub>50</sub> for inhibition of lipid peroxidation (nM)	Octanol:PBS partition coefficient
Compound 4	210 ± 58	7.37 ± 1.56
Bromo Vit E	45 ± 26	33.1 ± 4.4
α-Tocopherol	36 ± 22	27.4 ± 1.0
Trolox	18500 ± 5900	N.D.
BrBTP	47000 ± 13000	3.83 ± 0.22

When mitochondria were exposed to oxidative stress compound 4 protected them against oxidative damage, measured by lipid peroxidation and protein carbonyl formation (Figure 4). This antioxidant protection was prevented by incubating mitochondria with the uncoupler FCCP to prevent uptake of compound 4, and lipophilic cations alone did not protect mitochondria (Figure 5). Most importantly, the uptake of compound 4 protected mitochondrial function, measured by the ability to generate a membrane potential, far more effectively than Vitamin E itself (Figure 6). This shows that the accumulation of compound 4 into mitochondria selectively protects their function from oxidative damage. In addition, we showed that compound 4 is not damaging to mitochondria at the concentrations that afford protection (Figure 7).

The next step was to determine whether compound 4 was accumulated by intact cells. Compound 4 was rapidly accumulated by intact 143B cells, and the amount accumulated was greater than that by ρ<sup>0</sup> cells derived from 143B cells. This is important because the ρ<sup>0</sup> cells lack mitochondrial DNA and consequently have far lower mitochondrial membrane potential than the 143B cells, but are identical in every other way, including plasma membrane potential, cell volume and protein content (Figure 8); this suggests that most of the compound 4 within cells is mitochondrial. A proportion of this uptake of compound 4 into cells was inhibited by blocking the plasma and mitochondrial membrane potentials (Figure 9). This energisation-sensitive uptake corresponds to an intra mitochondrial concentration of

compound 4 of about 2- 4 mM, which is sufficient to protect mitochondria from oxidative damage. These concentrations of compound 4 are not toxic to cells (Figure10).

Further details of the experimental procedures and results are discussed below.

Figure 4 shows the protection of mitochondria against oxidative damage by compound 4. Mitochondria were exposed to oxidative stress by incubation with iron/ascorbate and the effect of compound 4 on oxidative damage assessed by measuring TBARS (filled bars) and protein carbonyls (open bars). Rat liver mitochondria (10 mg protein) were incubated at 25°C in a shaking water bath in 2 ml medium containing 100 mM KCl, 10 mM Tris, pH 7.7, supplemented with rotenone (1.33 µg/ml), 10 mM succinate, 500 µM ascorbate and other additions. After preincubation for 5 min, 100 µM FeSO<sub>4</sub> was added and 45-55 min later duplicate samples were removed and assayed for TBARS or protein carbonyls.

Figure 5 shows a comparison of compound 4 with vitamin E and the effect of uncoupler and other lipophilic cations. Energised rat liver mitochondria were exposed to tert-butylhydroperoxide and the effect of compound 4 (filled bars), α-tocopherol (open bars), compound 4 + 333 nM FCCP (stippled bars) or the simple lipophilic cation bromobutyl triphenylphosphonium (cross hatched bars) on TBARS formation determined. Rat liver mitochondria (4 mg protein) were incubated in 2 ml medium containing 120 mM KCl, 10 mM Hepes-HCl pH 7.2, 1 mM EGTA at 37°C in a shaking water bath for 5 min with various additions, then tert butyl hydroperoxide (5 mM) was added, and the mitochondria incubated for a further 45 min and then TBARS determined.

Figure 6 shows how compound 4 protects mitochondrial function from oxidative damage. Energised rat liver mitochondria were incubated with iron/ascorbate with no additions (stippled bars), 5 µM compound 4 (filled bars), 5 µM α-tocopherol (open bars) or 5 µM TPMP (cross hatched bars), and then isolated and the membrane potential generated by respiratory substrates measured relative to control incubations in the absence of iron/ascorbate. Rat liver mitochondria were incubated at 25°C in a shaking water bath in 2 ml medium containing 100 mM KCl, 10 mM Tris, pH 7.7, supplemented with rotenone (1.33 µg/ml), 10 mM succinate, 500 µM ascorbate and other additions. After preincubation for 5 min, 100 µM FeSO<sub>4</sub> was added and after 30 min the incubation was diluted with 6 ml ice-cold STE 250 mM sucrose, 10 mM Tris-HCL (pH 7.4) and 1 mM EGTA, pelleted by centrifugation (5 min at 5,000 x g) and the pellet resuspended in 200 µl STE and 20 µl (= 1 mg protein) suspended in 1 ml 110 mM KCl, 40 mM HEPES, 0.1 M EDTA pH 7.2 containing

1  $\mu$ M TPMP and 50 nCi/ml [ $^3$ H] TPMP either 10 mM glutamate and malate, 10 mM succinate and rotenone, or 5 mM ascorbate/100  $\mu$ M TMPD with rotenone and myxothiazol (2  $\mu$ g/ml), incubated at 25°C for 3 min then pelleted and the membrane potential determined as above and compared with an incubation that had not been exposed to oxidative stress.

5 Figure 7 shows the effect of compound 4 on the membrane potential (filled bars) and respiration rate of coupled (open bars), phosphorylating (stippled bars) and uncoupled mitochondria (cross hatched bars), as a percentage of values in the absence of compound V. The effect of various concentrations of compound 4 on the membrane potential of isolated mitochondria was determined from the distribution of [ $^3$ H] TPMP by incubating rat liver  
10 mitochondria (2 mg protein/ml) in 0.5 ml medium as above containing 1  $\mu$ M TPMP and 50 nCi/ml [ $^3$ H] TPMP at 25°C for 3 min. After the incubation mitochondria were pelleted by centrifugation and the [ $^3$ H] TPMP in the supernatant and pellet quantitated by scintillation counting and the membrane potential calculated assuming a volume of 0.5  $\mu$ l/mg proteins and that 60% of intramitochondrial TPMP is membrane bound. To measure the effect of  
15 compound 4 on coupled, phosphorylating and uncoupled respiration rates, mitochondria (2 mg protein/ml) were suspended in 120 mM KCl, 10 mM Hepes-HCl pH 7.2, 1 mM EGTA, 10 mM K Pi in a 3 ml Clark oxygen electrode then respiratory substrate, ADP (200 $\mu$ M) and FCCP (333 nM) were added sequentially to the electrode and respiration rates measured.

Figure 8 shows the uptake of compound 4 by cells. Here  $10^6$  143B cells (closed  
20 symbols) or  $p^0$  cells (open symbols) were incubated with 1  $\mu$ M [ $^3$ H] compound 4 and the compound 4 accumulation ratio determined. Human osteosarcoma 143B cells and a derived  $p^0$  cell line lacking mitochondrial DNA were cultured in DMEM/10 % FCS (foetal calf serum) supplemented with uridine and pyruvate under an atmosphere of 5% CO<sub>2</sub>/95% air at 37°C, grown to confluence and harvested for experiments by treatment with trypsin. To measure  
25 [ $^3$ H] compound 4 accumulation cells ( $10^6$ ) were incubated in 1 ml HEPES-buffered DMEM. At the end of the incubation, cells were pelleted by centrifugation, the cell pellet and the supernatant prepared for scintillation counting and the accumulation ratio [compound 4/mg protein]/(compound 4/ $\mu$ l)] calculated.

Figure 9 shows the amount of compound 4 taken up by  $10^6$  143B cells over 1 h  
30 incubation, corrected for inhibitor-insensitive binding. Human osteosarcoma 143B cells were incubated in 1 ml HEPES-buffered DMEM with 1-50  $\mu$ M compound 4 supplemented with 6-60 nCi/ml [ $^3$ H] compound 4. To determine the energization-dependent uptake, parallel

incubations with 12.5  $\mu$ M oligomycin, 20  $\mu$ M FCCP, 10  $\mu$ M myxathiazol, 100 nM valinomycin and 1mM ouabain were carried out. At the end of the incubation, cells were pelleted by centrifugation and prepared for scintillation counting and the energisation-sensitive uptake determined.

5           Figure 10 shows the effect of compound 4 on cell viability. Here, confluent 143B cells in 24 well tissue culture dishes were incubated with various concentrations of compound 4 for 24 h and cell viability measured by lactate dehydrogenase release.

10           Figure. 11 shows the UV-absorption spectrum of [10-(6'-ubiquinonyl) decyltriphenylphosphonium bromide] (herein referred to as "the quinone form of compound 1" or "mitoquinone") and of the reduced form of the compound [10-(6'-ubiquinonyl)decyltriphenylphosphonium bromide] (herein referred to as "the quinol form of compound 1" or "mitoquinol").

15           Figures 12A to 12D show reactions of [10-(6'-ubiquinonyl) decyltriphenylphosphonium bromide (1)] ("mitoquinone") and the reduced form of the compound ("mitoquinol") with mitochondrial membranes. Beef heart mitochondrial membranes (20 mg/ml) were suspended in 50 mM sodium phosphate, pH 7.2 at 20°C. In panel A rotenone and antimycin were present and for the t=0 scan, then succinate (5 mM) was added and scans repeated at 5 minute intervals as indicated. In panel B A<sub>275</sub> was monitored in the presence of rotenone and antimycin and then mitoquinone (50 mM) was added, followed by succinate (5 mM) and malonate (20 mM) where indicated. In Panel C rotenone, 20 ferricytochrome c (50 mM) and malonate (20 mM) were present, A<sub>275</sub> was monitored and mitoquinol (50 mM) and myxathiazol (10 mM) were added where indicated. In panel D, A<sub>550</sub> was monitored and the experiment in Panel C was repeated in the presence of KCN. Addition of myxathiazol inhibited this rate by about 60-70%. There was no reaction between 25 mitoquinone and succinate or NADH in the absence of mitochondrial membranes, however mixing 50 mM mitoquinone, but not mitoquinol, with 50 mM ferricytochrome c led to some reduction of A<sub>550</sub> ;

30           Figure 13 shows reactions of mitoquinol and mitoquinone with pentane-extracted mitochondrial membranes. Pentane extracted beef heart mitochondria (100 mg protein/ml) were suspended in 50 mM sodium phosphate, pH 7.2 at 20°C. In Panel A NADH (125 mM) was added and A<sub>340</sub> was monitored and ubiquinone-1 (UQ-1; 50 mM) added where indicated. This was repeated in Panel B, except that mitoquinone (1) (50 mM) was added. In Panel C pentane extracted mitochondria were incubated with mitoquinone (1) (50 mM), A<sub>275</sub> was

monitored and succinate (5 mM) and malonate (20 mM) added where indicated. In Panel D pentane-extracted mitochondria were incubated with NADH (125 mM), ferricytochrome c (50 mM) and  $A_{550}$  was monitored and mitoquinone (1) (50 mM) was added where indicated. Addition of myxathiazol inhibited the rate of reduction by about 60-70%;

5 Figure 14 shows reduction of mitoquinone (1) by intact mitochondria. Rat liver mitochondria (100 mg/ml) were incubated in 120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.2 at 20°C. and  $A_{275}$  monitored. In Panel A rotenone and succinate (5 mM) were present and mitoquinone (1) (50 mM) was added where indicated. This experiment was repeated in the presence of malonate (20 mM) or FCCP (333 nM). In Panel B glutamate and malate (5  
10 mM of each) were present from the start and mitoquinone (50 mM) was added where indicated. This experiment was repeated in the presence of FCCP or with rotenone and FCCP. Addition of TPMP (50 mM) instead of mitoquinone did not lead to changes in  $A_{275}$  ;

Figure 15 shows uptake of radiolabelled mitoquinol by energized rat liver mitochondria and its release on addition of the uncoupler FCCP; and

15 Figure 16 shows the effect of mitoquinol on isolated rat liver mitochondria. In Panel A rat liver mitochondria energized with succinate were incubated with various concentrations of mitoquinol and the membrane potential determined as a percentage of control incubations. In Panel B the respiration rate of succinate energized mitochondria under state 4 (black), state 3 (white) and uncoupled (stippled) conditions, as a percentage of control incubations.

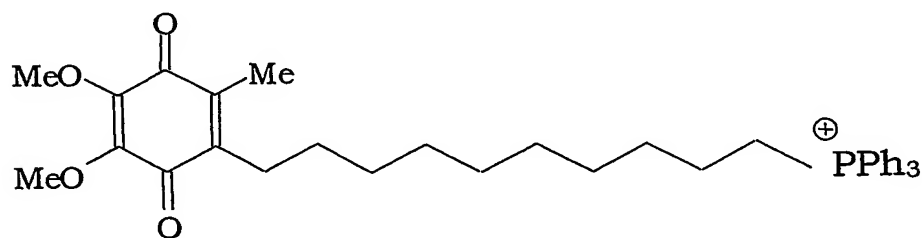
20

### EXAMPLE 5.

#### Anti-oxidant efficacy of exemplary mitochondrially-targeted compound mitoquinone/ol (compound 1)

A further exemplary compound is depicted in the following formula

25



Compound 1

which may exist as either the oxidized form (depicted) or as the quinol.

Stock solutions of the quinone in ethanol were stored at  $-80^{\circ}\text{C}$ . in the dark and their concentrations confirmed by  $^{31}\text{P}$  nmr. The compound was converted to the fully oxidized form by incubation in basic 95% ethanol over an hour on ice or by incubation with beef heart mitochondrial membrane at room temperature, either procedure leading to the same extinction coefficient of  $10,400\text{ M}^{-1}\text{ cm}^{-1}$  at the local maximum of 275 nm, with shoulders at 263 and 268 nm corresponding to the absorption maxima of the triphenylphosphonium moiety (Smith, R. A. J., Porteous, C. M., Coulter, C. V., and Murphy, M. P. 1999 *Eur J Biochem* **263**, 709. Burns *et al.*, 1995, *Arch Biochem Biophys* **332**, 60) and a broad shoulder at 290 nm due to the quinol (Crane *et al.*, 1971 *Meth. Enzymol*, **18C**, 137). Reduction by addition of  $\text{NaBH}_4$  gave the spectrum of the quinol which had the expected peak at 290 nm with an extinction coefficient of  $1800\text{ M}^{-1}\text{ cm}^{-1}$  and the extinction coefficient for at 268nm was  $3,000\text{ M}^{-1}\text{ cm}^{-1}$  the same as that for the phosphonium moiety alone (Burns *et al.*, 1995, *Arch Biochem Biophys* **332**, 60). The extinction coefficient of  $10,400\text{ M}^{-1}\text{ cm}^{-1}$  at 275 nm was lower than that for other  $\square\text{oxidize}\square$  which have values of  $14,600\text{ M}^{-1}\text{ cm}^{-1}$  in ethanol (Crane *et al.*, 1971 *Meth. Enzymol*, **18C**, 137) and  $12,250\text{ M}^{-1}\text{ cm}^{-1}$  in aqueous buffer (Cabrini *et al.*, 1981 *Arch Biochem Biophys*, **208**, 11). While the absorbance of the quinone was about 10% lower in buffer than in ethanol, the discrepancy was not due to an interaction between the phosphonium and the quinone as the absorbance of the precursor quinone before linking to the phosphonium and that of the simple phosphonium methyltriphenylphosphonium were additive when 50 mM of each were mixed together in either ethanol or aqueous buffer. The  $\Delta\epsilon_{\text{ox-red}}$  was  $7,000\text{ M}^{-1}\text{ cm}^{-1}$ .

The spectrum of fully oxidized mitoquinone (1) (50 mM) in 50 mM sodium phosphate, pH 7.2 is shown in Figure 11. Addition of  $\text{NaBH}_4$  gave the fully reduced compound, mitoquinol. The UV absorption spectrum of the reduced (quinol) and oxidized (quinone) forms of compound (1) are shown in Figure 11. To determine whether the mitochondrial respiratory chain could also  $\square\text{oxidize}\square$  or reduce compound 1 was incubated with beef heart mitochondrial membranes (Figure 12). In Panel A the spectrum of fully oxidized quinone form of compound (1) in the presence of antimycin inhibited membranes is shown ( $t=0$ ; Figure 12A). Addition of succinate led to the gradual reduction of the quinol form of compound (1) as measured by repeating the measurement every five minutes and showing that the peak at 275 nm gradually disappeared, the presence of antimycin prevented the oxidation of the quinol by mitochondrial complex III. Succinate did not lead to the complete reduction of the quinone form of compound (1) to the quinol form, as can be seen by comparing the complete reduction brought about by borohydride (Figure 11), instead it

reduced about 23% of the added ubiquinone (Figure 12A). This is presumably due to equilibration of the quinol/quinone couple with the succinate/fumarate couple ( $E_m Q=40$  mV at pH 7,  $E_m \text{ Suc}=30$  mV), hence this proportion corresponds to an  $E_h$  of about +8 mV.

The reduction of quinone form of compound (1) can be followed continuously at  $A_{275}$  nm (Figure 12B). On addition to rotenone inhibited mitochondrial membranes the small amount of the quinol form of compound (1) remaining was oxidized leading to a slight increase in  $A_{275}$ , but on addition of the Complex II substrate succinate the quinone form of compound (1) was rapidly reduced and this reduction was blocked by malonate, an inhibitor of Complex II (Figure 12B). The rate of reduction of the quinone form of compound (1) was  $51 \pm 9.9$  nmol/min/mg protein, which compares with the rate of reduction of cytochrome c by succinate in the presence of KCN of 359 nmol/min/mg. Allowing for the 2 electrons required for reduction of the quinone form of compound (1) compared with 1 for cytochrome c the rate of electron flux into the compound (1) pool is of similar order to the electron flux through the respiratory chain.

To determine whether the quinol form of compound (1) was oxidized by Complex III of the respiratory chain, the quinol form of compound (1) was added to beef heart membranes which had been inhibited with rotenone and malonate (Figure 12C). The quinol form of compound (1) was oxidized rapidly by membranes at an initial rate of about  $89 \pm 9$  nmol mitoquinol/min/mg protein (mean of  $2 \pm$  range) and this oxidation was blocked by myxathiazol an inhibitor of complex III (Figure 12C). To confirm that these electrons were being passed on to cytochrome c, the quinol form of compound (1) was then added to membrane supplemented with ferricytochrome c and the rate of reduction of cytochrome c monitored (Figure 12D). Addition of the quinol form of compound (1) led to reduction of cytochrome c at an initial rate of about  $93 \pm 13$  nmol/min/mg (mean  $\pm$  range). This rate was largely blocked by myxathiazol, although a small amount of cytochrome c reduction (about 30-40%) was not blocked by myxathiazol.

Compound (1) may be picking up and donating electrons directly from the active sites of the respiratory complexes, or it could be equilibrating with the endogenous mitochondrial ubiquinone pool. To address this question the endogenous ubiquinone pool was removed from beef heart mitochondria by pentane extraction. In the absence of endogenous ubiquinone as an electron acceptor the pentane extracted beef heart mitochondria could not oxidize added NADH, but addition of ubiquinone-1, a ubiquinone analog that can pick up electrons from the active site of complex I, the oxidation of NADH is partially restored

(Figure 13A). Similarly, addition of the quinone form of compound (1) also restored NADH oxidation indicating that the quinone form of compound (1) can pick up electrons from the complex I active site (Figure 13B). Succinate could also donate electrons to the quinone form of compound (1) in pentane extracted beef heart mitochondrial in a malonate sensitive manner, suggesting that the quinone form of compound (1) could also pick up electrons from the active site of Complex II (Figure 13C). Finally, the effect of the quinone on the flux of electrons to cytochrome c was determined and it was shown that there was no NADH-ferricytochrome c activity until the quinone form of compound (1) was added (Figure 13D), and this was partially inhibited by myxathizol (60-70%).

The next step was to see if the quinone form of compound (1) also accepted electrons within intact mitochondria (Figure 14). When the quinone form of compound (1) was added to intact energized mitochondria it was rapidly reduced (Figure 14A). In the presence of the uncoupler FCCP to dissipate the membrane potential the rate was decreased about 2-3 fold, presumably due to the prevention of the uptake of the compound in to the mitochondria (Figure 14A). The complex II inhibitor malonate also decreased the rate of reduction of the quinone form of compound (1) (Figure. 14A). Use of the NADH-linked substrates glutamate/malate also led to the rapid reduction of the quinone form of compound (1) by intact mitochondria which again was decreased by addition of the uncoupler FCCP (Figure 14B). The Complex I inhibitor rotenone also decreased the rate of reduction of the quinone form of compound (1) (Figure 14B).

A tritiated version of compound (1) was made in order to determine whether the quinol form was accumulated by energized mitochondria. This was incubated with energized mitochondria and the amount taken up into the mitochondria determined. It can be seen that the compound is accumulated rapidly and that this accumulation is reversed by addition of the uncoupler FCCP (Figure 15).

To determine the toxicity to isolated mitochondria and cells the effect on membrane potential and respiration rate were measured (Figure 16). It can be seen from Figure 16 that 10 mM mitoquinol had little effect on mitochondrial function and at 25 mM and above there was some uncoupling and inhibition of respiration.

#### **EXAMPLE 6.**

##### **Anti-oxidant efficacy of exemplary mitochondrially-targeted compound (2)**



The effect of compounds 1 or 2 on oxidative damage was assessed by measuring TBARS as described in Example 4 above and shown in Figure 4. Briefly, Rat liver mitochondria (10 mg protein) were incubated at 25°C in a shaking water bath in 2 ml medium containing 100 mM KCl, 10 mM Tris, pH 7.7, supplemented with rotenone (1.33 µg/ml), 10 mM succinate, 500 µM ascorbate and other additions. After preincubation for 5 min, 50 µM FeCl<sub>2</sub> was added and 45-55 min later duplicate samples were removed and assayed for TBARS fluorometrically ( $\lambda_{\text{excite}} = 515 \text{ nm}$ ;  $\lambda_{\text{emission}} = 553 \text{ nm}$ ).

Isolated mitochondria were exposed to oxidative stress by incubation with iron/ascorbate. As can be seen in Figure 17, the antioxidant efficiency of compound (2) is in the same order as that observed for compound (1), thereby demonstrating the antioxidant efficacy of the antioxidant compounds of the present invention.

## INDUSTRIAL APPLICATION

The compounds of the invention have application in selective antioxidant therapies for human patients to prevent mitochondrial damage. This can be to prevent the elevated mitochondrial oxidative stress associated with particular diseases, such as Parkinson's disease or diseases associated with mitochondrial DNA mutations. They could also be used in conjunction with cell transplant therapies for neurodegenerative diseases, to increase the survival rate of implanted cells.

In addition, these compounds could be used as prophylactics to protect organs during transplantation, or ameliorate the ischaemia-reperfusion injury that occurs during surgery.

The compounds of the invention could also be used to reduce cell damage following stroke and heart attack or be given prophylactically to premature babies, which are susceptible to brain ischemia. The methods of the invention have a major advantage over current antioxidant therapies – they will enable antioxidants to accumulate selectively in mitochondria, the part of the cell under greatest oxidative stress. This will greatly increase the efficacy of antioxidant therapies. Related lipophilic cations are being trialed as potential anticancer drugs and are known to be relatively non-toxic to whole animals, therefore these mitochondrially-targeted antioxidants are unlikely to have harmful side effects.

Those persons skilled in the art will appreciate that the above description is provided by way of example only, and that different lipophilic cation/antioxidant combinations can be employed without departing from the scope of the invention.

**ABSTRACT**

The invention provides mitochondrially targeted antioxidant compounds. A compound of the invention comprises a lipophilic cation covalently coupled to an antioxidant moiety. In preferred embodiments, the lipophilic cation is the triphenyl phosphonium cation, and the compound is of the formula  $P(Ph_3)^+XR \cdot Z^-$  where X is a linking group, Z is an anion and R is an antioxidant moiety. Also provided are pharmaceutical compositions containing the mitochondrially targeted antioxidant compounds, and methods of therapy or prophylaxis of patients who would benefit from reduced oxidative stress, which comprise the step of administering the compounds of the invention.

10

DATED THIS 22<sup>nd</sup> DAY OF August  
AJ PARK 2003  
PER *[Signature]*  
AGENTS FOR THE APPLICANT

Intellectual Property  
Office of NZ

22 AUG 2003

RECEIVED

Figure 1

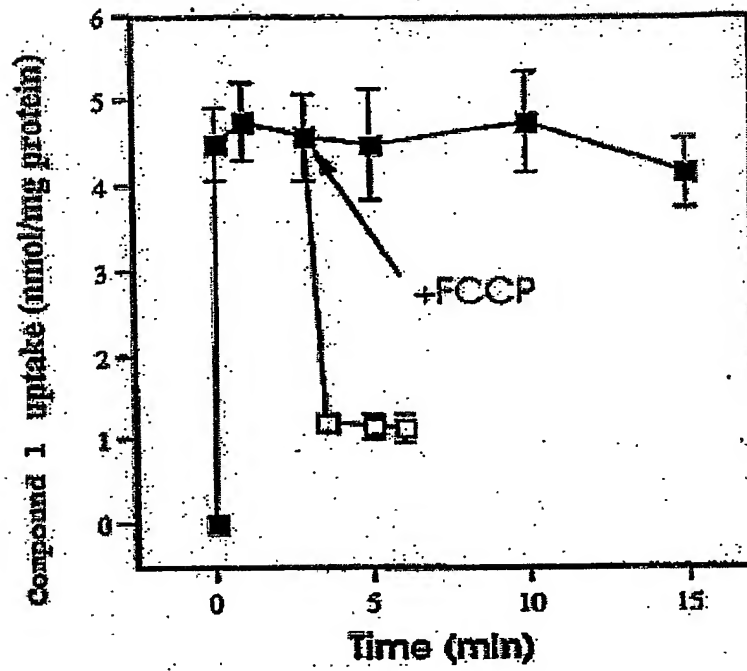
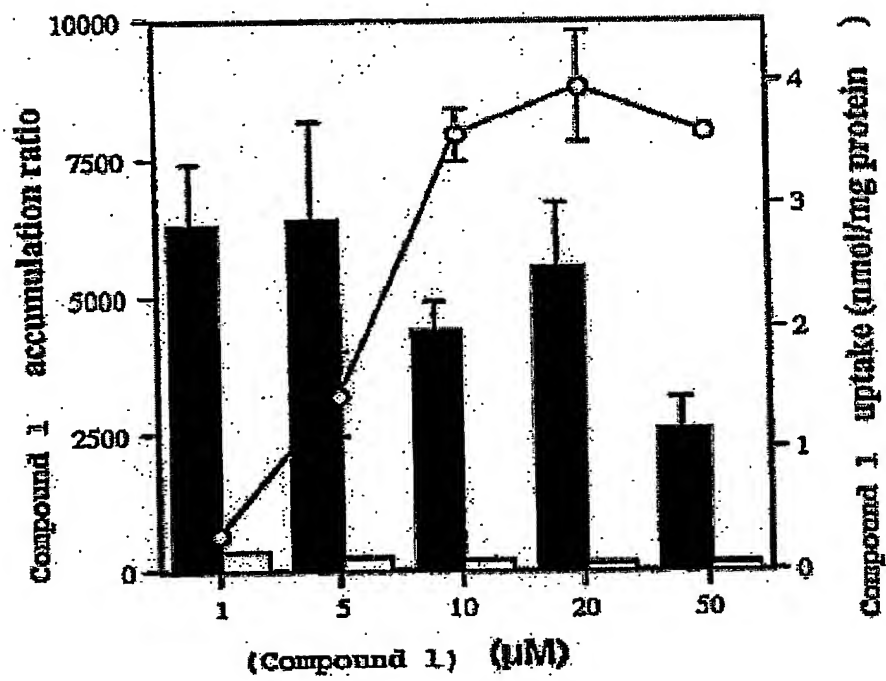


Figure 2



3/18

Figure 3

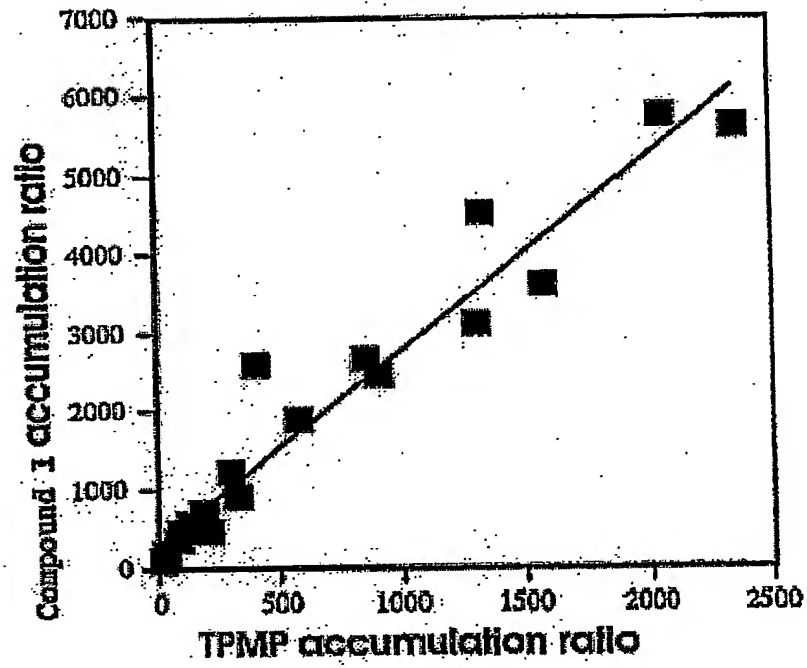
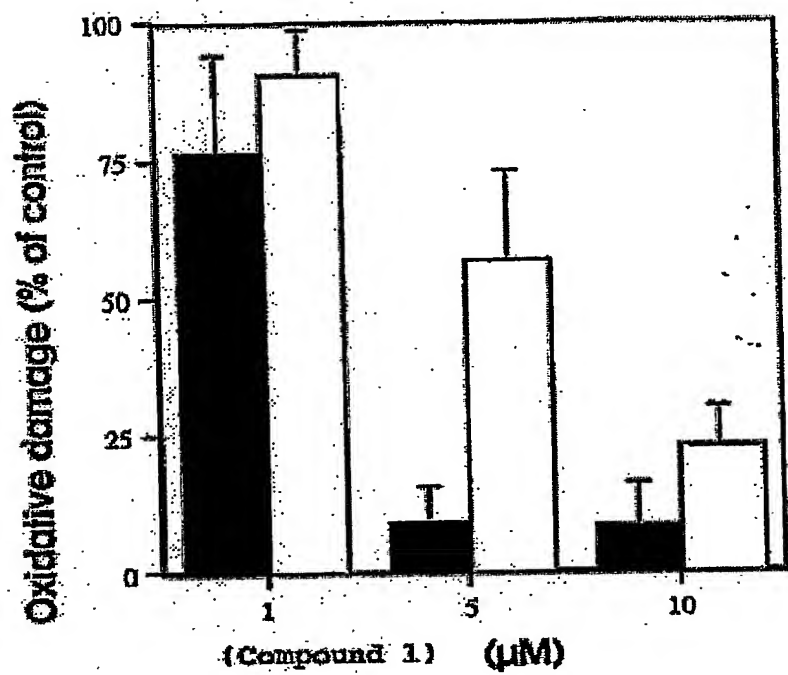


Figure 4



5/18

Figure 5

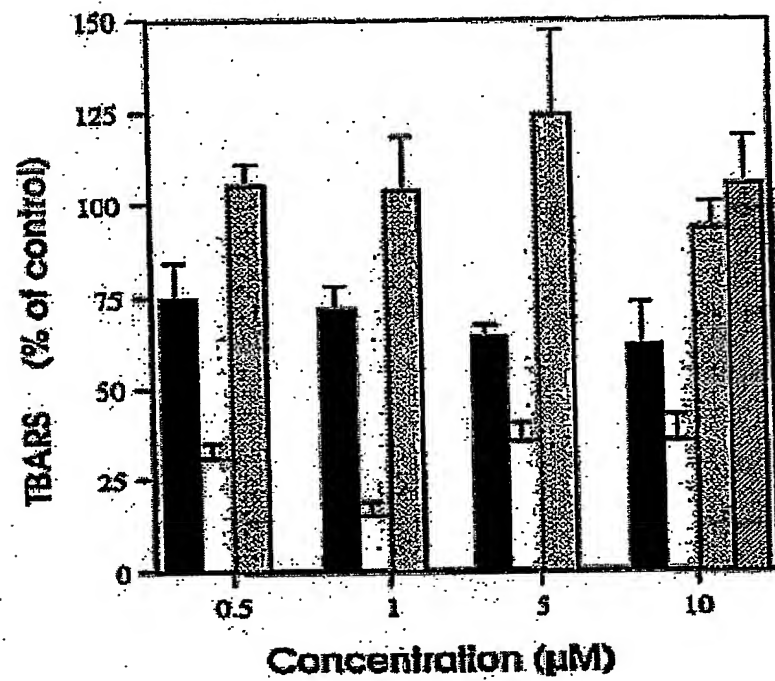
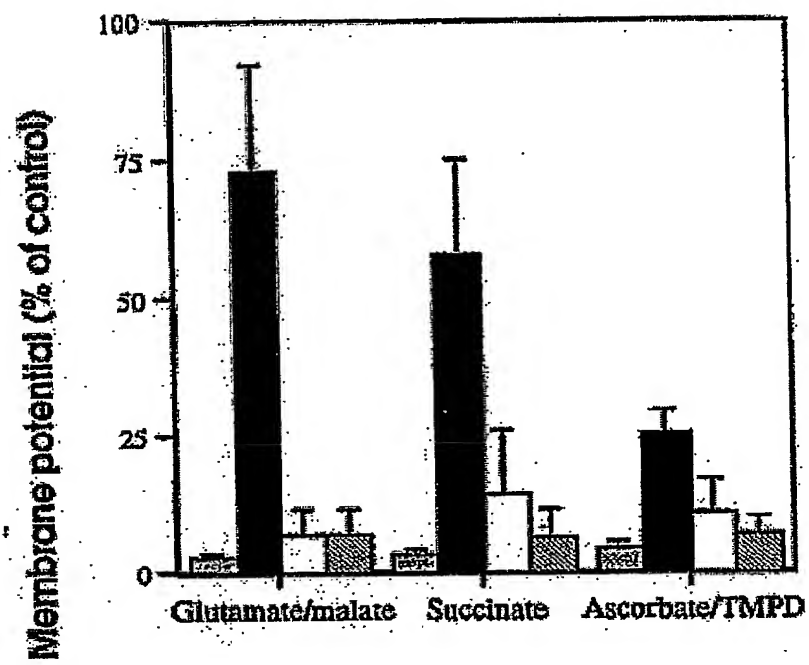


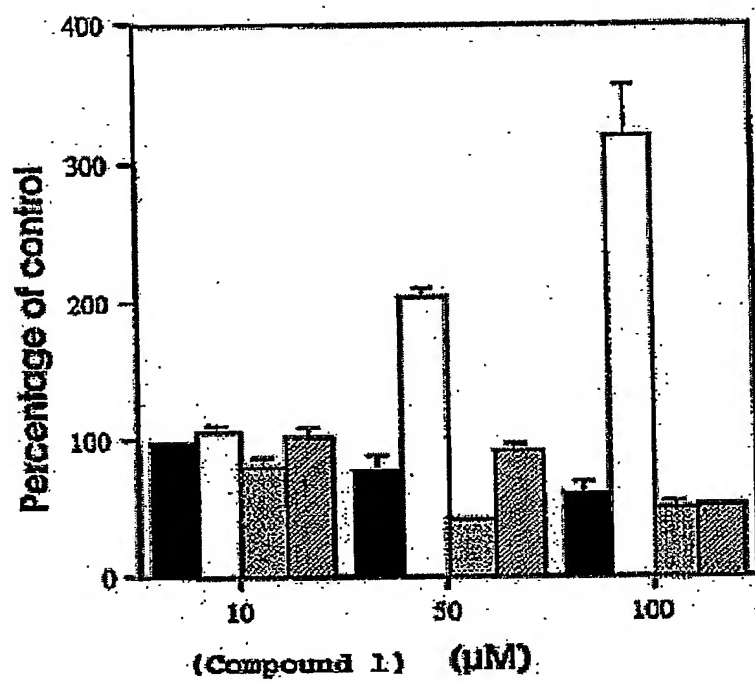


Figure 6



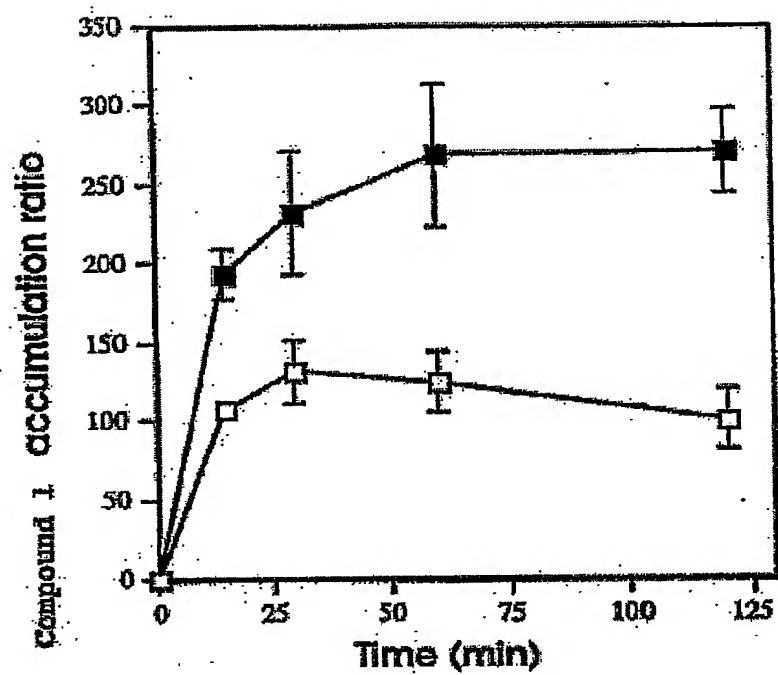
7/18

Figure 7



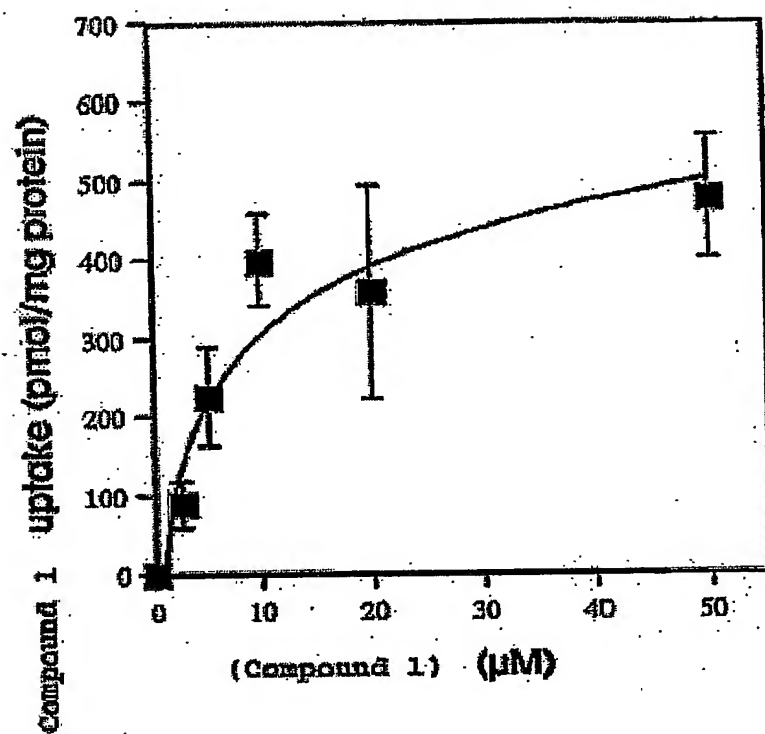
8/18

Figure 8



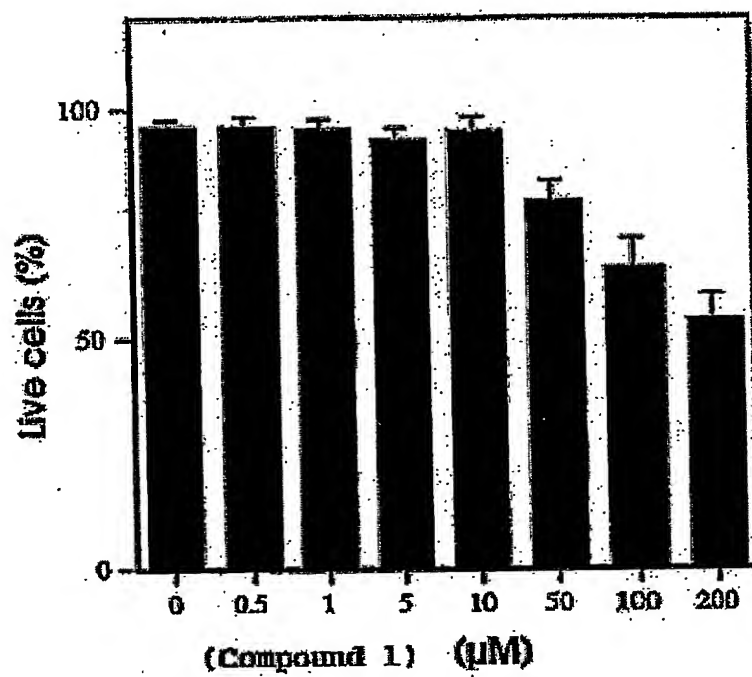
9/18

Figure 9



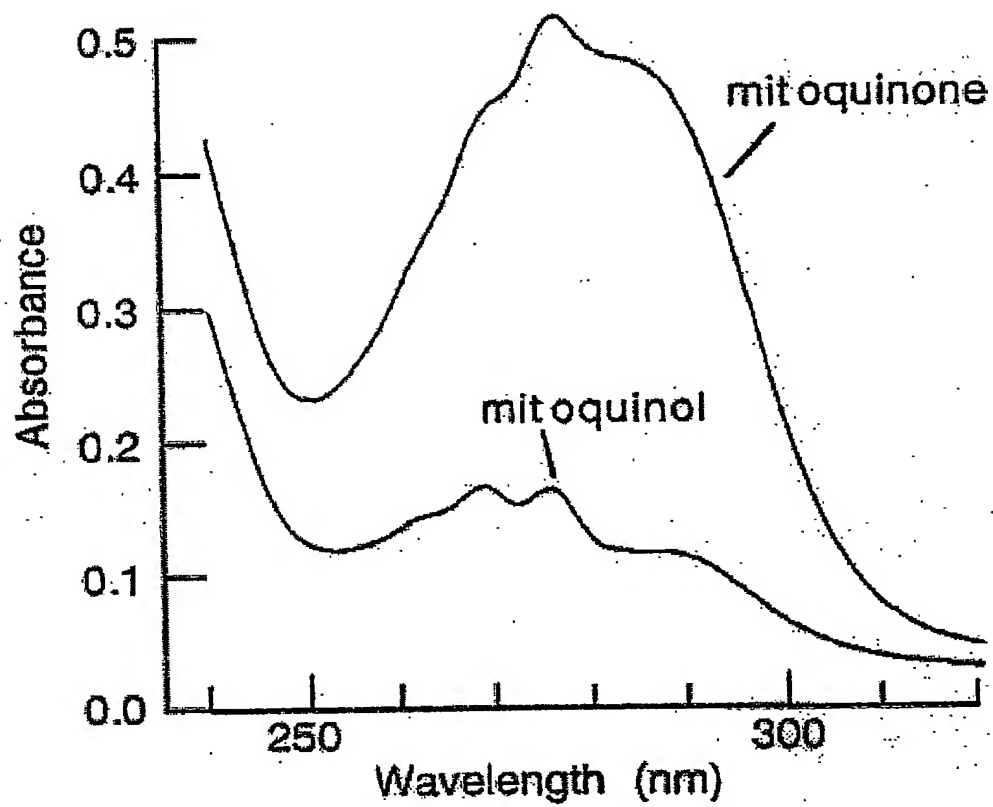
10/18

Figure 10



11/18

Figure 11



12/18

Figure 12

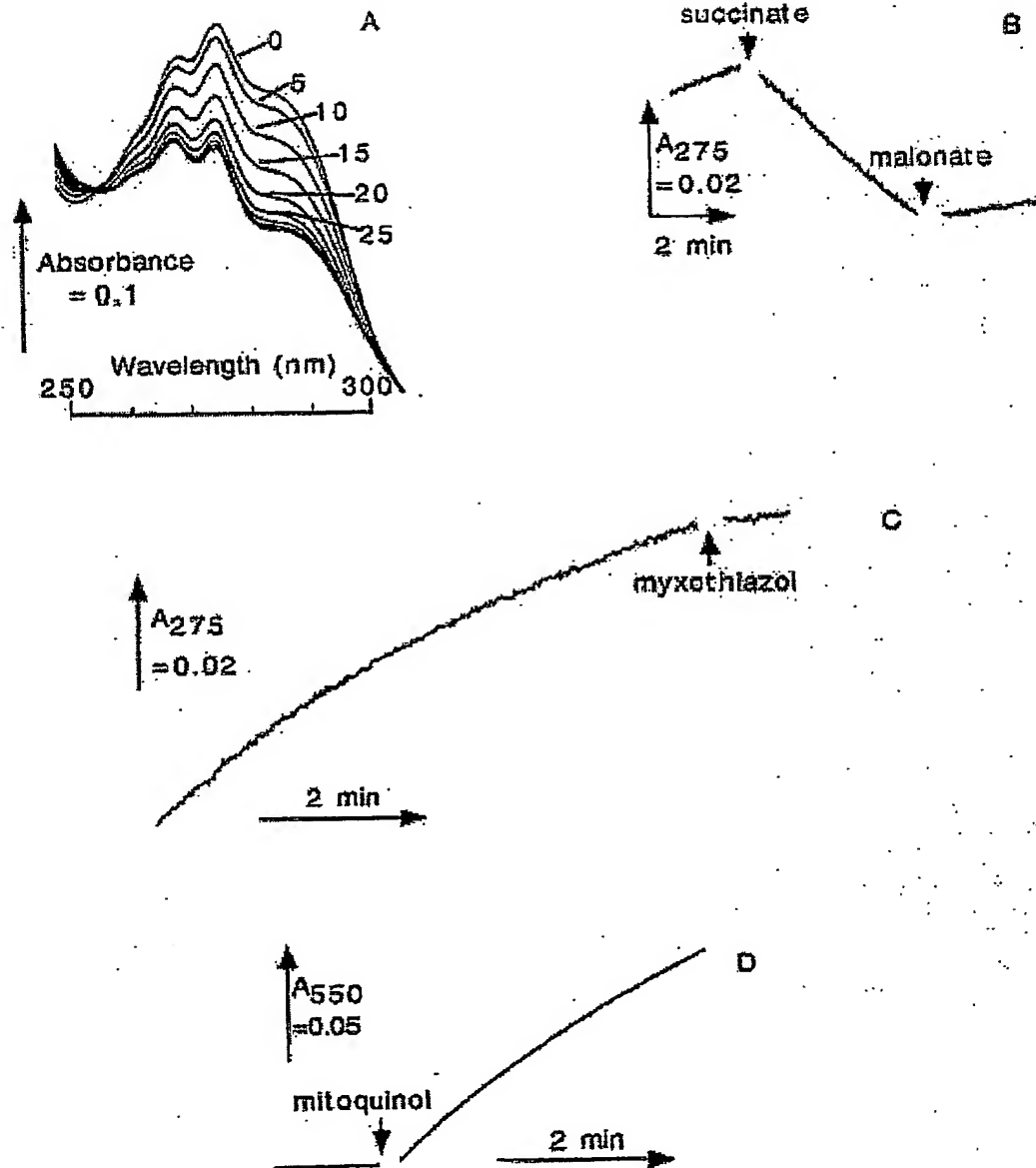


Figure 13

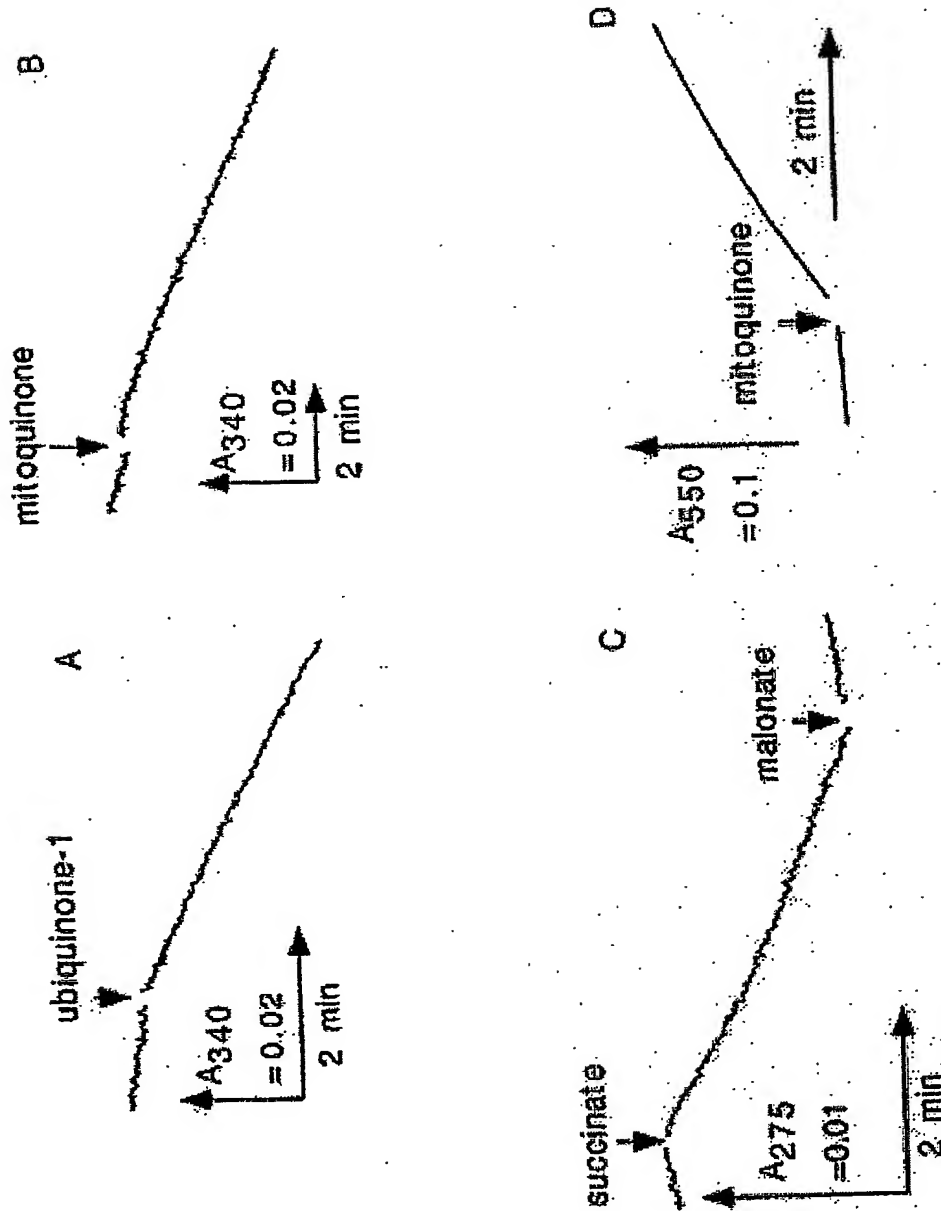
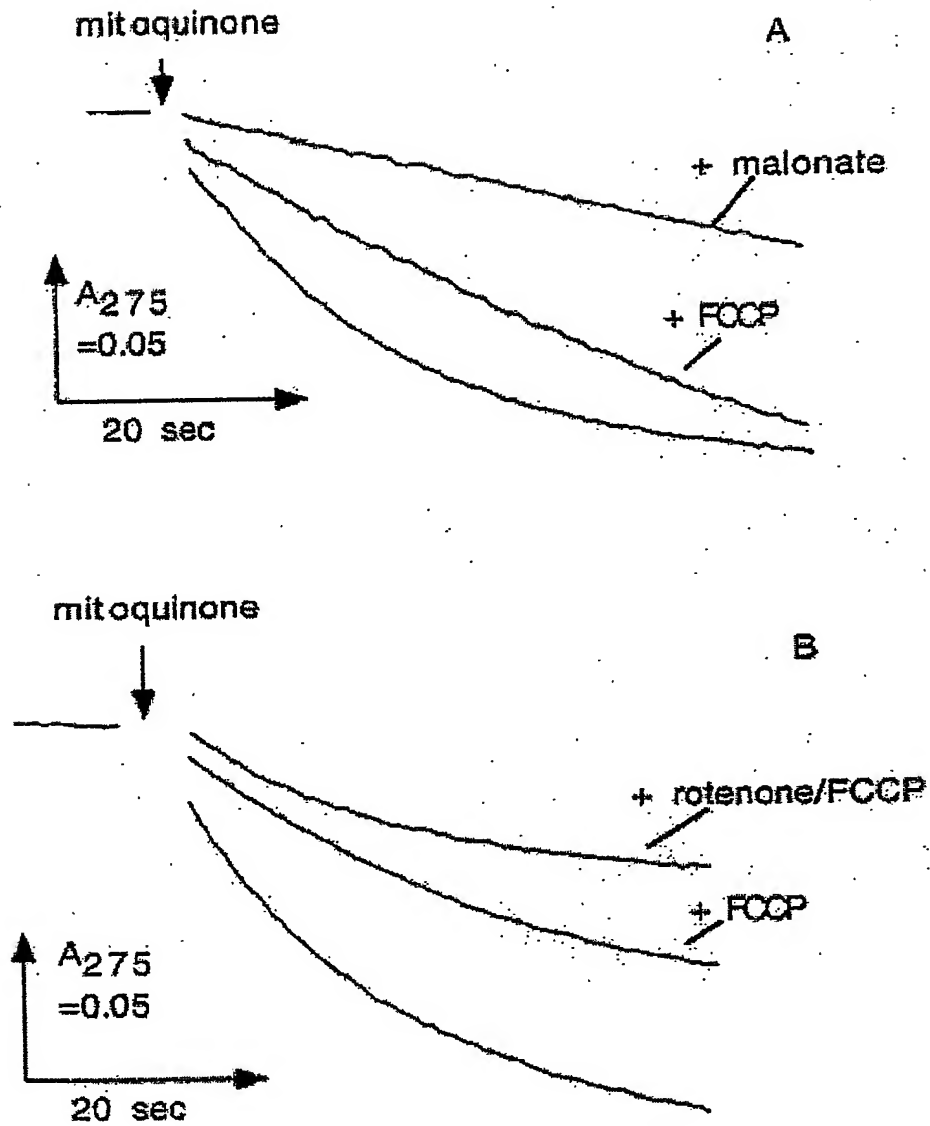




Figure 14



15/18

Figure 15

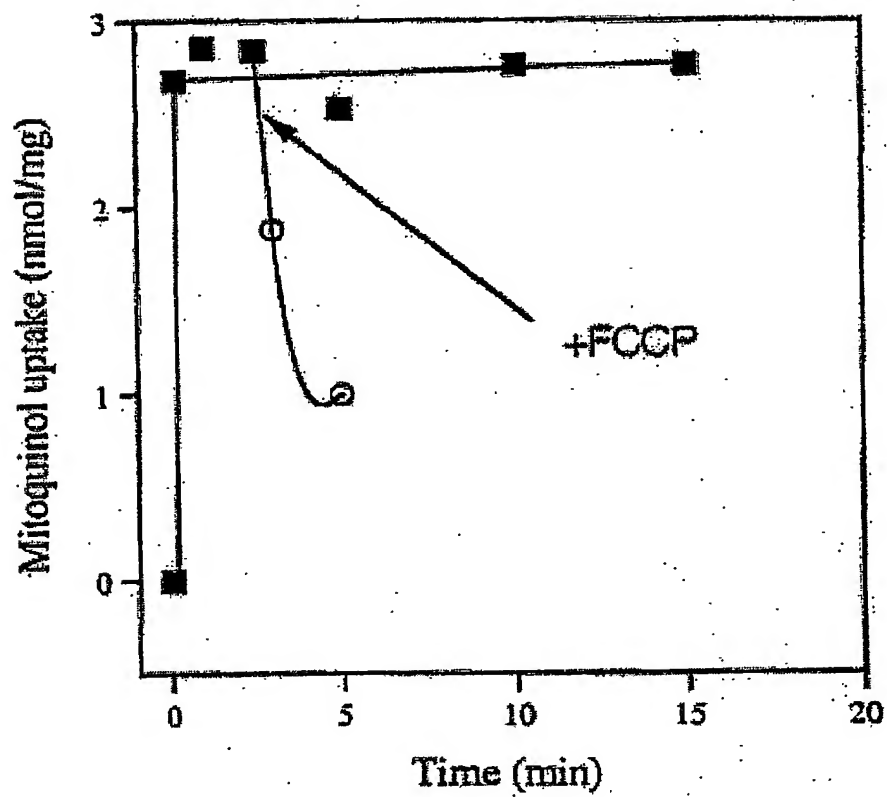
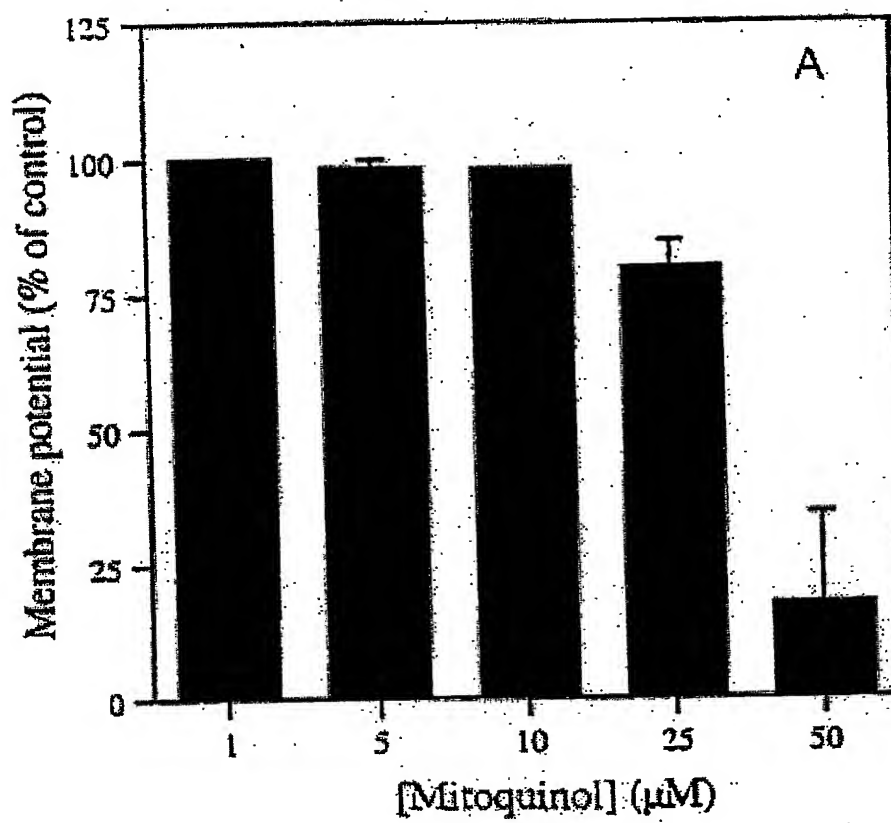
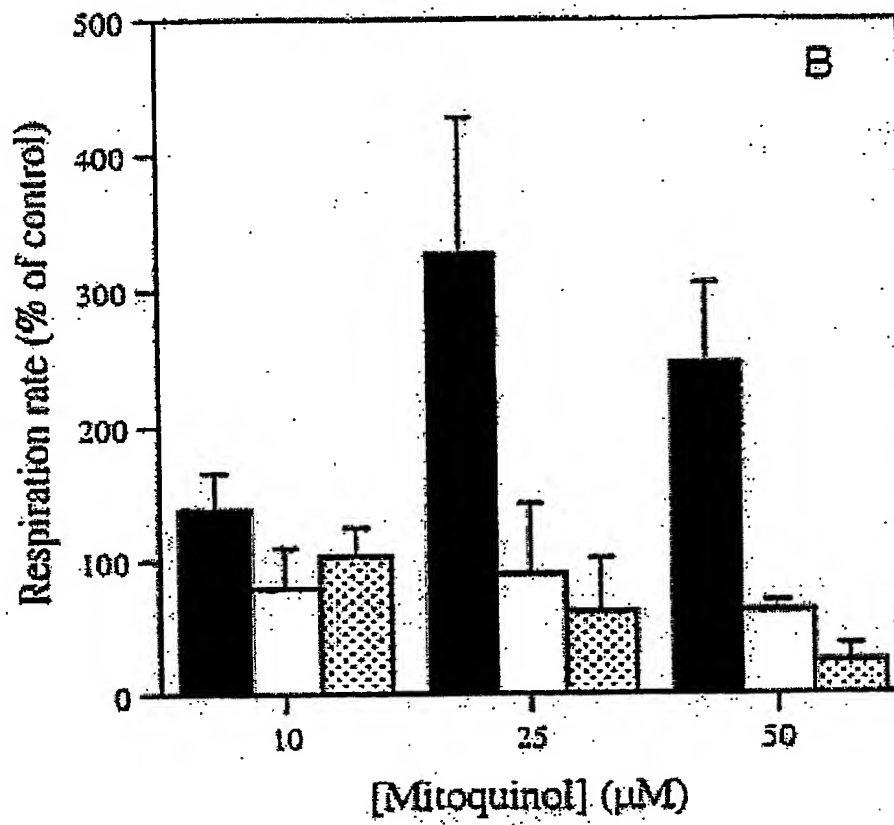


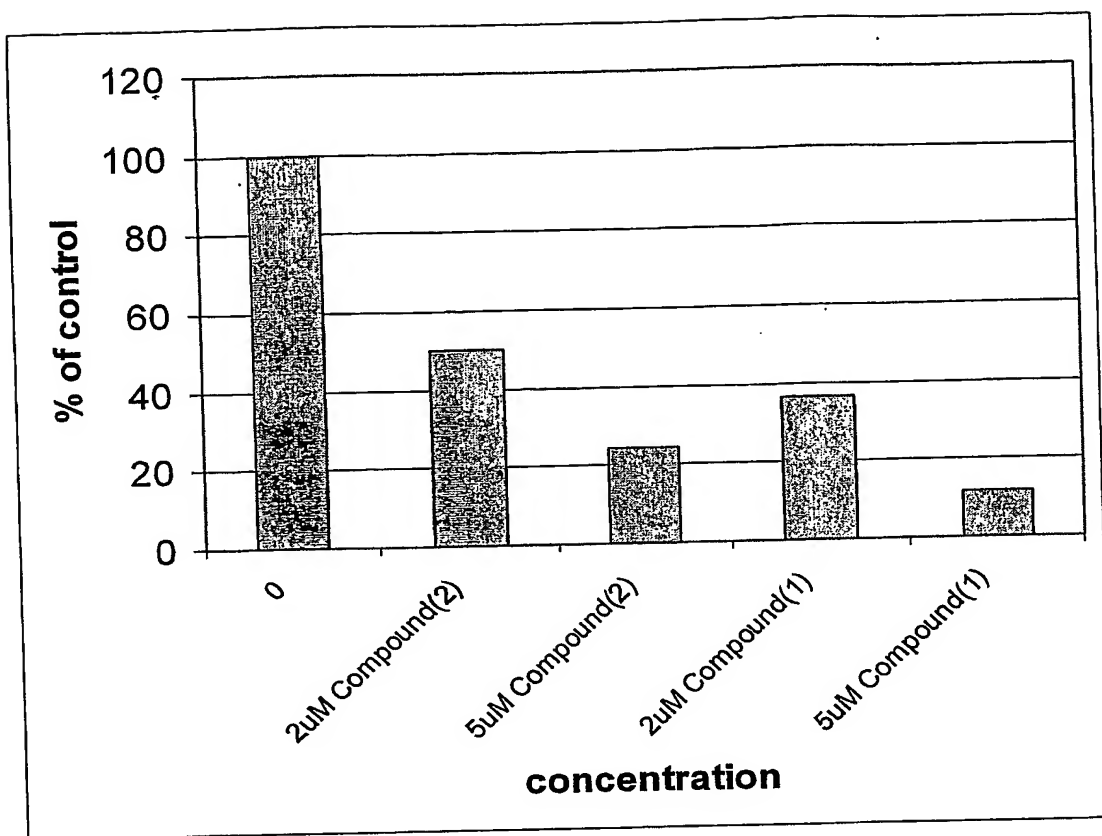
Figure 16A



17/18

Figure 16B





**Figure 17**

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ BLACK BORDERS

☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES

☐ FADED TEXT OR DRAWING

☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING

☒ SKEWED/SLANTED IMAGES

☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS

☐ GRAY SCALE DOCUMENTS

☒ LINES OR MARKS ON ORIGINAL DOCUMENT

☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**